MICROSTIMULATORS AND MICROTRANSDUCERS FOR FUNCTIONAL NEUROMUSCULAR STIMULATION

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ABSTRACT

We are developing a new class of implantable electronic devices for a wide range of neural prosthetic applications. Each implant consists of a microminiature capsule that can be injected into any desired location through a 12 gauge hypodermic needle. Multiple implants receive power and digitally-encoded command signals from an RF field established by a single external coil. The first type of implant is a single-channel microstimulator equipped with a capacitor that can store charge electrolytically and release it upon command as current-regulated stimulation pulses. We are also working on implants equipped with bidirectional telemetry that can be used to record sensory feedback, motor command, and/or internal control, signals and transmit them to the external control system.

In this quarter, we concentrated on implementing the various process improvements that were identified in the last quarter to be critical for improving the yields and confirming the reliability of the hermetic seals. The new ASICs for both the BION stimulator and the transceiver to test outgoing telemetry continue to be delayed by newly discovered layout problems. We report on the results of a pilot study in which we examined the combined effects of intramuscular stimulation and testosterone therapy on cat skeletal muscles.

In the past quarter, we successfully completed preclinical regulatory testing of the implant materials and submitted a journal article describing long-term active testing of our previous generation of microstimulators in cats. We are still refining the hermetic sealing and testing procedures for the current generation microstimulator, which has required additional fixturing and process control to improve reliability and yields and to reduce operator skill in preparation for conventional manufacturing. A successful series of hermetically sealed units was made just prior to the mailing of this report. A new method for humidity sensing is described. The new integrated circuits for an improved microstimulator with higher compliance voltage and a transceiver test chip for back telemetry were released to the foundry. We hope the new designs will fix a newly discovered latch-up problem discovered in the old IC chip. Various improvements have been made to the RF coil-driver circuits and to the test accessories for the bedside controller.

Implantable Devices

Upgrades to CO2 laser

Experience has shown that the exact location, size and strength of the beam are critical, but that physical motion of the beam is unnecessary during the weld (the parts themselves are in rotating chucks). Various improvements have been made to the stability and reproducibility of the optical path, particularly in the vertical direction (see below) and to provide finer control of beam power (replacing 8 bit DAC with 12 bit

DAC). We have also reworked the collets to improve the centering of the parts as they are rotated.

Ta electrode and capsule seal

The new Ta slugs with the scratch-free wire arrived and are now being used successfully, producing hermetic capsule subassemblies with yields greater than 90% (tested at $<2 \times 10^{-11}$ cc atm He/s). (The first batch had to be replaced by the slug manufacturer after we discovered scratches that had gotten onto the wire during their pressing and sintering process.) These seals have an excellent physical profile and photo elastic stress pattern. No further development of this process is anticipated.

Pt-Ir Feedthrough

We have now obtained Pt-Ir tubes precut to the proper length. We then tumble these and the Pt-Ir and Ir washers in fine abrasive to get to the exact diameters needs for the slip-fits that are important for the YAG laser welds between them. Calibration procedures have been defined for the YAG laser to improve the reproducibility of these low-power welds. The tumbling also produces a satin finish which seems to produce more consistent and better appearing seals to the glass.

A new mathematical review of this seal has indicated a potential source of thermal mismatch which is presently being studied and will be reported on in the future.

We were puzzled by the appearance of very narrow bands of high stress on the outside surface of the glass beads after the bead-to-tube CO2 laser weld. These were traced to reflection from the Pt-Ir tube and focussing by the molten glass, which acts somewhat like a lens. This problem was solved by shifting the beam off-axis so that it struck the glass bean tangentially rather than straight on. This, however, increased the precision needed on beam size and positioning and parts centering (see above) because small changes greatly affect the heat transferred to the glass bead.

The capsule closing seal (glass capillary to glass bead) is now working reliably, with a yield of 22 of 24 attempted on our last batch (hermeticity checked through the open Pt-Ir tube at $<2 \times 10^{-11}$ cc atm He/s). A consistent pattern of compressive stress is present at the glass-tube interface, with very low levels of stress on the outside surfaces of the glass bead and capillary. The weld profile is slightly bell-shaped but should still fit in the lumen of the insertion tool. There is no detectable discoloration or loss of temper in the gold-plated elgiloy spring that connects the Pt-Ir washer to the μPCB.

Final Seal

The final seal (closing the hollow Pt-Ir feedthrough) should be simple and reliable, given the long experience in the implanted device industry with similar metal-metal final seals. We have had some problems with this, perhaps because we have not paid as much attention to it. These have included dropping the plug wire too deeply into the electronics, damaging the previously made glass-to-tube seals with mechanical shock

and thermal stress, and uneven melting of the plug leaving gross leaks. These appear to have been solved by the present strategy, which includes scoring and cutting the protruding tube with a very sharp end-cutter, plugging the tube with a slightly oversized ball melted onto the end of a short length of pure Pt wire, and heat-sinking aggressively to the surface of the Ir electrode during a rapidly pulsed YAG weld directly on the center of the plug. A multiple fixture is now being built to permit us to run a sufficient number of units to obtain a yield figure.

Start of soak tests

Six working, sealed BIONs were anodized and bomb-tested (see below). These units had no getters and no vacuum bake-out. Three showed no leaks and three had possible gas bubbles coming from seals (it is difficult to be certain because of the possibility of trapped gas in the electrodes and exterior metal-metal welds themselves). These were sent to Queen's U. where they have been started on active stimulation and temperature cycling, with a cycle of 3 hours at 35◦C and 9 hours at 85◦C, with continuous stimulation in 5X hypertonic saline at 10 mA, 258 μs pulses at 50 pps. Results will be reported in the next quarter.

External Equipment

Inventory

We now have on hand 10 working bedside controllers, 2 working handheld coils and drivers, 5 working drywells with preamplifiers, 2 working single-chamber wetwells,

and 1 working 8-chamber wetwell. The large animal coils and drivers have been returned to IIT for retuning in light of a latch-up problem that prevents about 2/3 of the units that work well with the handheld coils from working consistently in the more uniform magnetic field of the large animal coils.

Insertion tool design

The present system uses a conventional Intracath™ modified so that the trochar can be connected by banana plug to a laboratory stimulator, with the indifferent electrode elsewhere on the patient's skin. This is adequate for our own testing, but not appropriate for a clinical product. The stimulation requirements are modest, but we must be sure that the waveforms are suitable for the material of the trochar to avoid electrochemical damage to the tissue. We are considering a disposable insertion tool that would incorporate a battery-operated stimulator in the handle to generate preset stimulation pulses with a modest range of amplitude adjustment. This would permit the clinician to operate the stimulator without assistance while performing the insertion procedure under aseptic conditions.

Preclinical Regulatory Testing

Testosterone study (Appendix)

While we are waiting for fabrication of sufficient working BIONs to complete our preclinical animal trials, we have conducted a pilot study of the effects of combining testosterone with electrical stimulation delivered through simulated BION electrodes. As

discussed in that report, there are reasons to believe that this combination may be particularly effective, particularly for dealing with atrophy in elderly patients who tend to have low levels of circulating testosterone.

Plans for Next Quarter

We will continue the chronic soak testing of the BIONs. We will decide on the mechanical configuration for the getter, which is intended to absorb water vapor that may leak in at rates undetectable by conventional leak-testing. We will redo the ASICs and submit for a MOSIS foundry run, suspending the AMI wafer run. We will complete implantation of the long-term cats with dummy BIONs. The results are described in the Appendix.

The potential thermal mismatch problem will be further reviewed in light of ongoing experiments.

I.C. Development at Pritzker Institute and Alfred E. Mann Foundation

During the last project period work at the Pritzker Institute has focused upon analysis and revision of the Repeater and Microstimulator Integrated Circuits, and a re-examination of our work scope for project year #3.

Integrated Circuit Layout Revisions

By way of review, both of these chip designs have seen a troubled past. The Repeater chip was designed to answer critical questions about inward and outward telemetry data rates. The Microstimulator chip was a redesigned version of the 2MHz microstimulator. Both of these chips were initially fabricated at the Australian Foundry AWAM to take advantage of their high voltage process. The results were less than satisfactory. Although one foundry run of the Repeater chip yielded valuable information from bench tests, no significant results were seen from two foundry runs of the Microstimulator chip. The precise causes of these nonproductive runs were caused by fabrication errors at AWAM. Apparently AWAM had not used their own test circuits to monitor their process. John Gord at AEMF detected these problems which we reported to AWAM. When we reported this to AWAM and insisted that they use their own test patterns to verify their process during fabrication, they admitted that personnel changes at their foundry had caused the problem. Not only our chip run, but a major run by Pacesetter was also affected by their problem. Pacesetter switched foundrys and AWAM recalled their representative from the United States.

Because of this experience, the decision was made, last year, to change from AWAM to another company with a high voltage process. Two different consulting layout individuals were used, one for the Microstimulator and one other for the Repeater chip. The performance of both of these layout consultants was unsatisfactory. Although the completed layouts involving a reticle with 16 microstim chips, 2 microtelemetry chips and a test component chip were submitted to the new foundry for fabrication in March of 1997, an examination of these chips, at IIT and AEMF, revealed disturbing errors in the layout files. Simultaneously, the foundry performed design-rule-checks which also identified certain layout errors. Some of these design-rule-check errors were due to errors in foundry's high voltage design-rule-check for high voltage circuits. At AEMF errors were found in the final design changes that were introduced by the second layout person. Apparently the second layout house that has previously performed excellently for AEMF in other projects, had undertaken too large of a work load with other customers and now had become inaccurate in their layout work. At this point the fabrication run was stopped.

A new layout house was found and corrections were made. To reduce the financial gamble (at the expense of time delay) the reticle was reduced in half and run through MOSIS. Efforts at IIT and AEMF focused upon verification of the layout-versus-schematic, and correcting design-rule-violations and preparing the files for MOSIS submission. This team effort worked well, and both designs were submitted to MOSIS on May 7, 1997. An error by the new layout house was recently found in the repeater chip by John Gord at

AEMF. We have corrected the layout error and, if possible, will try to submit the corrected repeater chip to MOSIS on the next run.

We expect the return of the fabricated chips in early to mid July, 1997. At IIT, we are preparing for bench testing of Repeater chip and an associated Test chip. A similar plan for the Microstimulator is being prepared at the Mann Foundation. Based upon those tests, decisions will be made as to the course of action for the next MOSIS submission date of July 30, 1997. In all probability revisions of these chips will be made at that time.

Transmitter Fabrication and Delivery by Pritzker Institute

Although the appropriate excitation and modulation parameters for the new AMI 2MHz microstimulator at not presently known, we have continued to support device testing taking place at Queens University. Several transmitters were returned to IIT so that we could determine the cause of intermittent behavior of the existing microstimulators. It was subsequently determined that a type of "latch-up" malfunction of the 2 MHz chips was responsible for the variable operation of the previously fabricated microstimulators. In order to provide Queens with some means of animal testing, we implemented the square-wave modulation on one of the cat-coil transmitters. It appeared that use of the square-wave modulation alleviated some of the latch-up for selected microstimulators. This transmitter has been returned to Queens for evaluation. This latch-up problem was originally missed due to the use of square wave transmitters constructed at AEMF, which did not reveal this problem.

Year 3 Work Scope

During the final year of this contract, IIT plans to turn its attention to items which were present in the original work scope, but for which the project has seen little progress. These include:

1. Perform goniometer experiments.

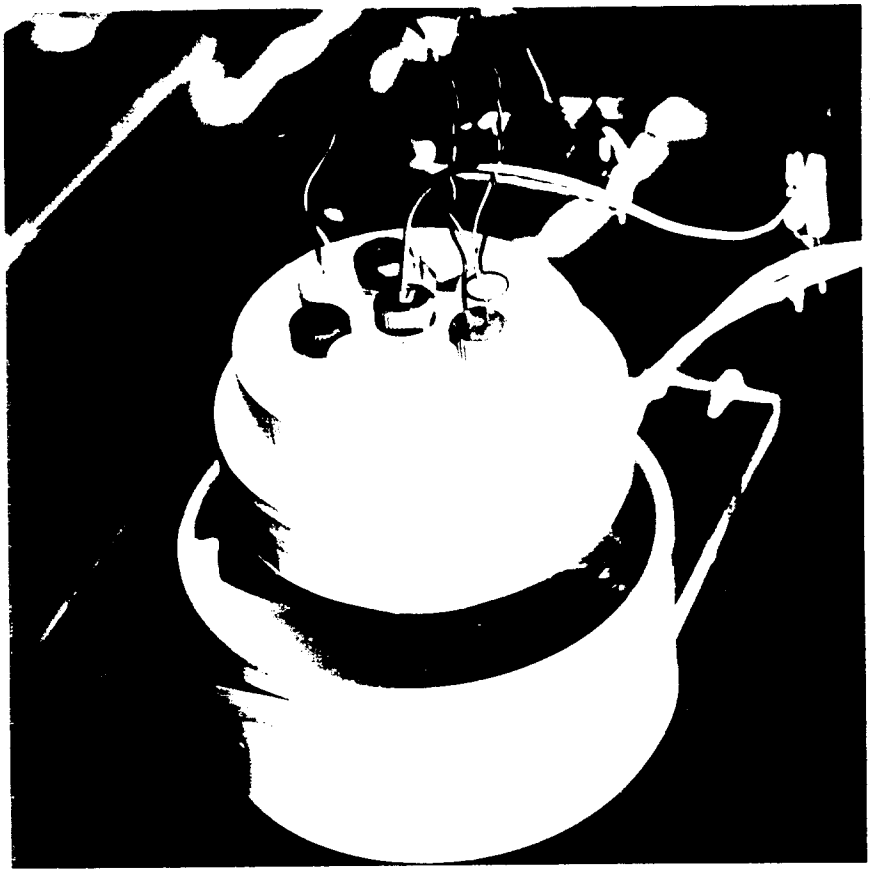
2. Design bandpass receiver circuitry in anticipation of repeater chip operation.

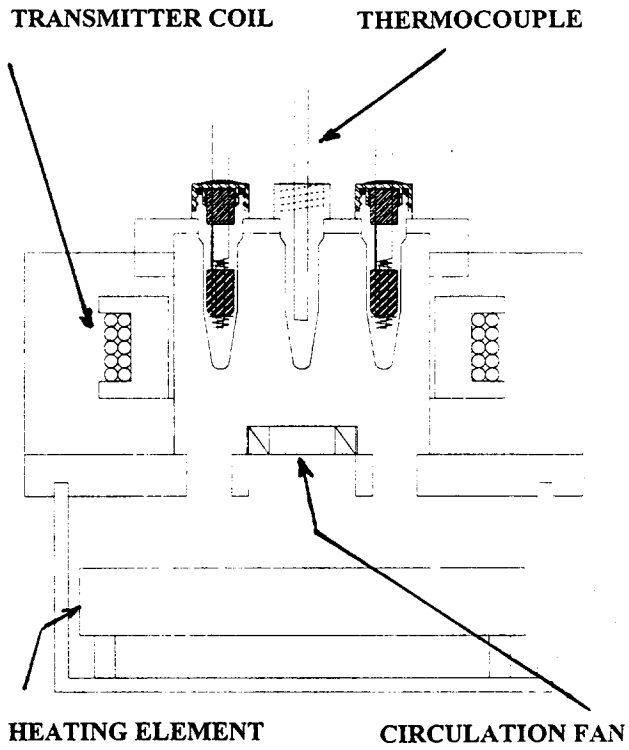
3. Test repeater chip and other delivered ASICs.

4. Moisture-test sealed micromodules

5. Design and fabricate wearable electronics once the specifications for such electronics can be established.

We are also preparing a block diagram for a combined sensor-telemetry/microstimulator integrated circuit that can be configured, by telemetry, to operate in either a sensor or stimulator mode and also telemeter out functional parameters for trouble shooting. We expect to complete a preliminary block diagram for this chip by the end of June, 1997.

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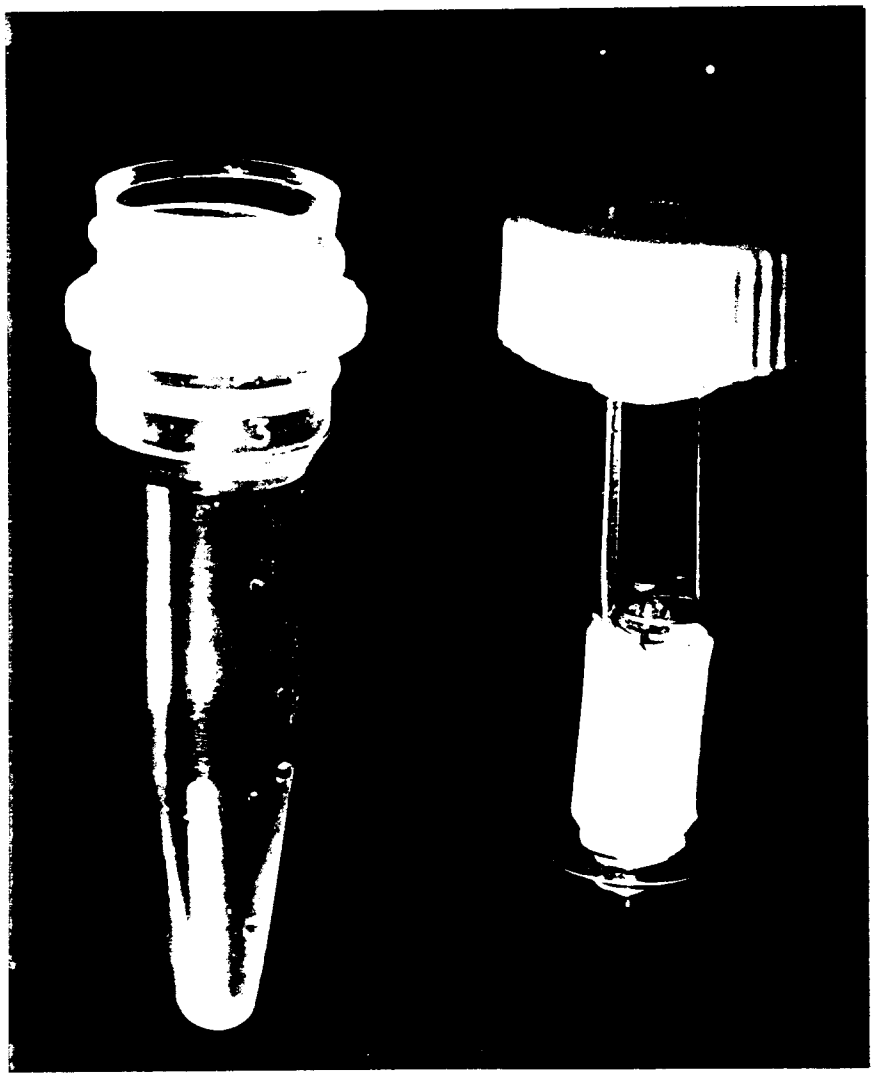

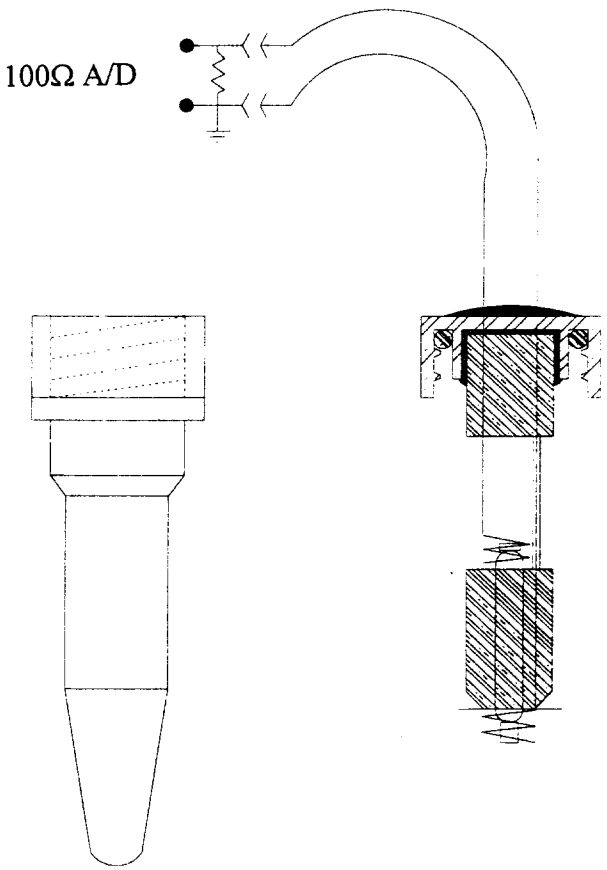
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The Effects of a Combination of Local Testosterone and Electrical Stimulation on Feline Skeletal Muscle

A thesis submitted to the Department of Physiology in partial fulfillment of the requirements for the degree of Bachelor of Science (Honours)

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ABSTRACT

A feasibility study was performed whose goal was to determine if a combination of local testosterone administration and electrical stimulation could induce hypertrophy of feline hindlimb skeletal muscles. The use of such stimulation has been proposed as a treatment for the muscle atrophy seen in many clinical conditions.

The failure to localize testosterone to the target muscles changed the study's focus to a determination of the effect of systemic testosterone and electrical stimulation. There were no significant increases in the weight of stimulated muscle compared to non-stimulated muscle. Upon examination of the depletion pattern of the stimulated muscles it was found that only a small percentage of the individual fibers were stimulated. The recruitment pattern of the method of electrical stimulation was found to differ from that of traditional applications of functional neuromuscular stimulation (FNS) in that the fibers primarily recruited were type I and type IIa as opposed to the type IIb fibers. In addition, there were significant increases in the cross-sectional areas of the stimulated type I and type IIa fibers compared to non-stimulated fibers in the same muscle.

These results may have profound clinical implications. Historically, the use of FNS in patients is complicated by the rapid fatiguing of the muscles being stimulated due to the recruitment of fast fatiguing type IIb fibers. The use of the intramuscular stimulation method used in this study may be able to recruit fatigue resistant type I and type IIa fibers thus alleviating the problem of fatigue in rehabilitating patients.

INTRODUCTION

Skeletal muscles are specialized tissues whose highly organized contractile proteins are designed to generate forces. They are remarkable for their plasticity that enables them to change their force-generating capabilities over a long period of time in the face of situational demands. Muscles that are challenged to generate very high forces generally grow stronger whereas muscle that is used minimally tends to atrophy and weaken.

The phenomenon of muscle atrophy can be defined as a decrease in the size of muscle fibers accompanied by a decrease in muscle mass. The functional consequence of muscle atrophy is weakening of the muscle. Muscle atrophy can be the result of several factors including denervation, disuse, or hormonal imbalances. For example, in patients who have suffered spinal cord injuries, the muscles that can no longer be activated by neural control undergo severe disuse atrophy. In women, the perineal muscles involved in micturition may undergo atrophy due to the hormonal changes that accompany menopause and these changes can result in urinary incontinence. Denervation caused by injury or neurodegenerative disease also results in muscle atrophy.

Muscle size can be increased by two methods. Increases in muscular work have been shown to increase muscle size and maximal tension development (Goldberg et al., 1975). Such increases can be achieved through exercise programs. In paralyzed or paretic muscle, the use of functional neuromuscular stimulation (FNS) to induce muscular contractions with electrodes has been shown to be effective in exciting muscle with intact peripheral innervation (Peckham et al., 1992).

Alternatively, the administration of testosterone has been suggested to produce muscle hypertrophy (Bhasin et al., 1996). However, the side effects of high circulating levels of exogenous testosterone include masculinization in females, menstrual irregularities, adverse effects on the liver, the promotion of aggressive behavior and testicular atrophy (Sherwood, 1989). To combat these problems, a testosterone pellet with local releasing characteristics has been developed to limit the amount of hormone entering the systemic circulation, yet ensure high local concentrations in the target muscles.

The purpose of this study was to determine the feasibility of a combined regimen of local testosterone administration and electrical stimulation for the reversal of muscle atrophy by inducing hypertrophy with a concomitant increase in muscle strength.

Effects of Exercise on Muscle

It is known that resistance exercise increases muscle size and strength as the active muscle fibers adapt in response to the demands being placed on them (Woo et al., 1987). Tenotomy studies, in which one of a pair of synergistic muscles is sectioned at the tendon so that the intact muscle is faced with an increased load, have shown significant increases in the size of the intact muscle (Goldberg et al., 1975). However, the mechanism by which muscles increase their size and strength is not completely understood.

One possible mechanism for muscle hypertrophy involves the release of trophic factors from nerves (Florini et al., 1996). The rate of release of these trophic factors from nerves may increase with high levels of stimulation, such as during exercise. Increased synthesis of contractile proteins may also be stimulated by damage to the muscle fibers induced by resistance training. Giddings et al. (1985) showed that weight lifting exercises performed by cats induced degenerative changes in muscle fibers. They also found that trained muscle had a larger proportion of large and small fibers in comparison to untrained muscle. Their results were interpreted as support for the idea that high resistance exercise can cause hypertrophy of individual fibers. Further, the results were seen to provide evidence for the notion that an increase in the number of fibers, or hyperplasia, could also occur as an alternative mechanism by which muscle hypertrophies. Historically, muscle hyperplasia has not generally been accepted as a contributing factor to increases in muscle size. However, recent studies (Gonyea et al., 1986) have shown that splitting of muscle fibers may occur in response to exercise and thus may contribute to muscle hypertrophy.

The Use of FNS to Stimulate Muscles

Functional neuromuscular stimulation (FNS) is a method of electrically stimulating muscles by applying current to the innervating motoneuron. It is used as a method of clinical treatment for individuals suffering from loss of voluntary motor control. This type of rehabilitation employs skin-surface or implanted electrodes which deliver electrical impulses to induce muscular contractions. The treatment has become increasingly popular for rehabilitating patients with spinal cord injuries and other conditions in which the motoneurons are intact but the command pathway is non-functional. FNS has been used successfully to exercise paralyzed lower limbs of spinal cord injured patients (Glaser, 1994), to stimulate hand and arm muscles for functional grasping, and it has also been used for standing and walking in disabled patients (Gordon et al., 1994).

Commonly, FNS systems involve the placement of an electrode at a site near the muscle entry point of a motor nerve. The electrode stimulates the nerve axon and initiates an action potential that is propagated down the axon to all of the motor terminals that the nerve supplies. The arrival of an action potential in the neuromuscular junction initiates a cascade of reactions that ultimately result in the contraction of the muscle innervated by the stimulated motoneuron (Sherwood, 1989). Thus, the mechanism of contraction elicited by FNS is the same as that associated with voluntary motor activity, except that the action potential is initiated by exogenous current pulses rather than central command pathways. All methods to produce electrical stimulation rely on a similar mechanism of operation in which the strength of contraction is governed by the size of electrical current pulses.

While FNS has been used successfully in a number of clinical applications, there are several inherent problems which prevent this method of rehabilitation from being used on a larger scale. The currently employed methods of FNS involve the use of implanted electrodes with percutaneous wires or transcutaneous electrodes. Percutaneous electrodes require extensive surgery for implantation and they leave the patient susceptible to infection at the site at which wires exit through the skin. Transcutaneous electrodes fail to stimulate deep muscles specifically and can cause skin irritation and pain through intact sensory nerve pathways. For these reasons, a fully implantable miniature stimulator that can receive its power and data through a RF field is currently being developed (Cameron et al., 1993). This type of device does not require invasive surgery and provides excellent specificity of the muscles it stimulates.

Previous studies examining the effects of electrical stimulation of muscle have shown that muscle mass and the percentage of protein content in the stimulated muscle can be made to increase under a variety of conditions. Inoue (1993) and colleagues showed, for example, that stimulation of rat gastrocnemius caused the cross-sectional areas in all of the fibers in the stimulated muscles to increase. The stimulation that was used was similar to that of training programs aimed at increasing muscle size in athletes. These programs typically involve three to four sets of six to eight repetitions with high resistance and long recovery periods in between sets. However, what set this study apart from many others with similar protocols was a second intriguing observation that the number of androgen receptors increased rapidly following the initiation of the electrical stimulation. The number of receptors was twenty-five percent higher in stimulated muscle compared to controls after three days of stimulation and they remained at that level for the remainder of the experimental period.

Effects of Androgens on Muscle

The superfamily of nuclear receptors that influence protein transcription includes androgen receptors. These receptors are intracellular, ligand-activated transcription factors. The binding of testosterone to these transcription factors result in the production of mRNA that translate into specific proteins.

The actions of testosterone are not equivalent in all target tissues. Androgens are sex hormones which act at many sites to ensure the development of secondary sexual characteristics and promote effective mating behaviors. Many studies have examined the effects of testosterone in sexually dimorphic muscles. The results have suggested that systemic testosterone treatment induces the synthesis of DNA in satellite cells of rat levator ani muscle (Joubert et al., 1995), and that local testosterone administration increases the weight of rat bulbocavernosus and levator ani muscles (Rand et al., 1992) while also

increasing the fiber diameters of treated muscles. In the frog, testosterone administration has been shown to prevent castration induced atrophy of muscles required for mating (Dorlochter et al., 1994).

The increased production of contractile proteins induced by testosterone results in greater cross-sectional area and more cross-bridge sites in parallel within each muscle fiber. Testosterone has been shown to increase muscle protein turnover, that is, the generation and the degeneration of muscle proteins (Urban et al., 1995). Such a finding implies that the synthesis of new proteins and the breakdown of less functional proteins initiated by testosterone can enhance muscle strength, enabling the muscle to develop higher maximal tensions.

While the cellular effects of testosterone are rather well understood, the consequences of testosterone administration in terms of its effects in the whole animal are less well known. Exogenous anabolic steroids have been used extensively by power athletes in attempts to increase skeletal muscle size and strength, even though many studies have failed to show that the hormones are actually effective for these purposes. Kuhn and colleagues (1985) demonstrated the lack of an anabolic effect of testosterone administration in overloaded muscles of female rats. The muscles of normal female rats had been shown to contain a greater concentration of cytosolic androgen receptors than normal males. For that reason, in addition to the fact that females have lower circulating levels of testosterone, Kuhn hypothesized that female rats would be more responsive to the actions of administered androgens than males. However, their investigation showed that testosterone administration in females had no effect on overloaded muscles despite the fact that the testosterone treatment elevated blood testosterone levels to twenty times their normal level.

Many of the studies on humans intended to determine the effects of testosterone have failed to show the results normally desired by athletes, such as increases in muscle size and strength. One of the possible reasons behind the failure of exogenous steroids to exhibit hypertrophic effects is that many studies use replacement doses of testosterone, such as those used in the treatment of hypogonadal men. The efficacy of testosterone as an anabolic agent may not have been demonstrated in many studies due, to either insufficient doses, or due to the fact that the receptors for the steroid may have been saturated. Since the use of electrical stimulation has been shown to increase the number of androgen receptors in muscles, it is conceivable that a combined regimen of electrical stimulation of muscles and high doses of testosterone would result in a significant increase in muscle size and strength. Furthermore, few studies have ever involved the control of exercise in combination with large doses of testosterone. A recent study on normal men who were given supraphysiological doses of testosterone in combination with a strict weight lifting regime showed that the combined effects on muscle size and strength are greater than the effects of testosterone or weight lifting alone (Bhasin et al., 1996).

Proposal

Traditionally, weight lifting or heavy resistance exercise has been used as a method of increasing muscle size and strength. High doses of exogenous testosterone have been used to amplify the gains obtained by exercise, but the use of androgens has been implicated in some unwelcome side effects because circulating androgens have effects on many of the body's tissues. To help to keep increases in circulating hormones to a minimum, testosterone pellets with local release characteristics have been developed commercially. These are claimed to produce high local concentrations of testosterone, 10 to 20 times higher within tissues 2.5 cm from the pellet, compared to circulating blood levels (Innovative Research of America, personal communication, Dr. Shafie).

It was the purpose of this research project to determine the efficacy of a combined regimen of locally delivered testosterone and electrical stimulation to induce muscle hypertrophy. These effects were determined by implanting stimulating electrodes and testosterone pellets into the hindlimb muscles of female cats and stimulating the muscles over a period of five weeks. The findings of this study will provide insight into the feasibility of such combined treatment to rehabilitate patients whose muscles have wasted from disuse atrophy because of spinal cord injuries or stroke. It is further intended as a first step in the development of systematic treatment of muscle atrophy which results in urinary incontinence in women.

METHODS

Training of the Experimental Animals

Prior to implantation, 2 female ovariectomized cats were trained so that they would become accustomed to the handling that they would receive during the post-implantation period. The training involved exposing the animals to the same procedures that were used during the post-implantation period. Positive re-enforcement was used to promote behavior desired during the stimulation trials.

Fabrication of the Stimulating and Dummy Electrodes and the "Backpack"

The muscles to be stimulated were activated by electrodes that reproduce the structure of recently developed BIONs. These devices were used to establish predicate experience with percutaneous electrodes against which the efficacy and ease of use of the RF-controlled devices could be compared. Each pair of stimulating electrodes was attached to a silicone tube, 24 mm in length and 2 mm in diameter (figure 1). Two Teflon coated, stainless steel wires were inserted into the tube. One wire pierced through the tube 4 mm from the entry site whereas the other came out 14 mm further along the tube. The wire coming out of the tube at 4 mm, designated as the anode, was wrapped around the tube twice. The other wire, designated as the cathode, was wrapped around four times. The tube was then filled with silicone gel which was allowed to cure. Dummy electrodes were identical to the stimulating electrodes.

A 6-pin connector was secured to a 5 cm by 5 cm piece of silastic sheeting using silicone. The connector and sheeting together was referred to as the "backpack" (figure 2).

Implantation of Stimulating Electrodes

On the day before the surgery, the animal was given a prophylactic antibiotic injection, 0.5 cc of Tribrissen (Coopers Agropharm Inc., Ajax, ON) sub-cutaneously.

On the day of surgery, the animal was weighed and anesthetized with sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON) at a dose of 35 mg/kg administered intraperitoneally. This anesthetic was chosen to prevent the animal from coming out of anesthesia too quickly during the 1 to 2 hour long surgical procedure and to ensure that the recovery period was long enough to allow the animal to begin to heal. Anesthetic level was frequently checked and maintained at a level which abolished the pedal reflex. When necessary, additional anesthetic was administered intravenously at a dose of 5 mg/kg.

The left and right hindlimbs, between the knee and the ankle, and the rump were shaved and cleaned. The animal was positioned on its belly and its hindlimbs were extended. A midline incision was made on the dorsal side of the lower left limb between the knee joint and the ankle joint to expose the medial and lateral gastrocnemia (MG, LG).

The nerves supplying both MG and LG were located. In each muscle, an electrode was implanted with the cathode closest to the nerve entry site by making a small incision in the distal part of the muscle and inserting the electrode through the incision (figure 3). A small suture was used to secure the device in place. The leads from the stimulating electrodes were slid sub-cutaneously through a hollow metal tube to a small incision site over the sacrum where the backpack was to be placed. The ends of the leads were brought through the skin and soldered in an ordered array to pins on the connector.

To administer testosterone in the vicinity of the stimulated muscle, a testosterone pellet (Innovative Research of America, Sarasota, FL.) designed to have a local releasing radius of 2.5 cm with a steady release rate over 60 days was placed in a 5 mm piece of silastic tubing and implanted into the connective tissue between MG and LG (figure 3) such that testosterone would be equally distributed to both muscles. The tubing was sutured to the connective tissue between the muscles and the skin incision was sutured. The implantation procedure was repeated on the right limb of the animal except that no testosterone pellet was implanted.

The animal was then turned onto its right side to allow the left hindlimb to be approached ventrolaterally. Using the tibial ridge as a landmark, an incision was made running from the knee to the ankle on the ventral side of the lower limb. This exposed both the tibialis anterior (TA) and extensor

digitorum longus (EDL) muscles. Once the site of nerve entry to these muscles had been located, an electrode was inserted into TA through a small distal incision in the muscle. A second electrode was inserted into EDL near the site of nerve entry (figure 4). Sutures were used to secure the devices in position. The leads from the stimulating electrodes were passed to the backpack using a hollow tube in the same way as leads from MG and LG. A second locally releasing testosterone pellet within a piece of silastic tubing was implanted and sutured to the connective tissue between TA and EDL. The incision was then sutured and the entire procedure was repeated on the right lower limb of the animal without the implantation of a testosterone pellet.

In one of the cats, the wire leads of the electrodes in left MG and TA were soldered onto the backpack. The remaining 6 electrodes served as dummy electrodes. In the second cat, the strategy was reversed so that the electrodes in left LG and EDL were connected to the backpack and actively stimulated while the other 6 electrodes remained inactive. Table 1 summarizes which muscles were implanted with what type of electrode.

Initially, the sheeting of the backpack was secured to the animal by four percutaneous sutures at the corners of the backpack. However, following recovery, irritation on the skin caused the animals to chew the backpack. Thus within one week of the initial surgery, the attachment of the backpack was revised by cutting the cutaneous sutures and passing a single 2-0 Ethibond suture through a hole drilled into a lumbar vertebral spine. This method of attachment was well tolerated by the animals. The point of attachment between the connector and the wires was covered with silicone in an attempt to prevent the cat from damaging the wires. When it was time to activate the electrodes, a biphasic Grass SD9 stimulator (Quincy, Mass.) was connected to the 6-pin connector.

Post Operative Care

Following surgery, the animal was given another prophylactic antibiotic injection. The animal was observed closely for any adverse reactions or unusual patterns of behavior. When the animal was able to swallow, water was given orally using a syringe. When the animal was able to take food, food was placed in the kennel. Notes of all procedures and the progress of recovery was recorded.

Threshold Measurements

Threshold measurements were begun three days after implantation. These measurements were designed to determine the strength of the stimulus required to produce contractions in the muscles implanted with active electrodes. During the first threshold test day, the animal was anesthetized lightly with an intramuscular injection of ketamine hydrochloride (9 mg/kg) and xylazine (0.5 mg/kg). During subsequent threshold measurements it was decided that anesthetic was not necessary. A biphasic Grass SD9 stimulator (Quincy, Mass.) was used to activate the electrodes through the pin connector on the backpack. Thresholds were obtained by setting the voltage amplitude to 0 and the pulse width to 200 msec. The voltage amplitude was increased slowly until a palpable muscle twitch was produced. The threshold was defined as the minimum voltage amplitude able to produce a palpable muscle twitch using a 200 msec pulse width. Threshold measurements for each muscle implanted with a stimulating electrode were repeated at weekly intervals. These determinations did not appear to be uncomfortable for the cat and thus subsequent measurements were made without anesthesia.

Force Measurements

The forces generated by the implanted muscles were measured weekly. To make this measurement, a cast instrumented with a strain gauge that fitted the ankle joint was created (figure 5). The cast was made from a sheet of Polyform Light (Smith & Nephew Rolyan Inc., Menomonee Falls, WI) which can be made pliable with heat so that it can be molded to the leg of a representative cat cadaver of similar size. Once cooled, the material becomes stiff and resistant to deformation. To add to the rigidity of the device, a curved aluminum rod was attached so that part of the rod would lie against the anterior aspect of the tibia while the other part would lie against the ventral surface of the tarsal bones. A strain

gauge was attached to the aluminum rod at the point where torsional forces would be exerted by flexion or extension around the ankle. The cast was placed onto the lower limb of the animal following threshold measurements. Any attempts at dorsiflexion or plantar flexion by the muscles of the calf were prevented by the cast, but the force exerted by the muscles was measured by the strain gauge. The output of the strain gauge was displayed using a Hitachi VC-6023 Digital Storage Oscilloscope.

For measurements on the muscles of the lower hindlimb, the animal was placed on the side that was not being measured and it was restrained lightly. The knee joint was firmly held by the experimenter to prevent any movement at the hip or at the knee during stimulation. The angle between the femur and the tibia was set at 150 degrees which gave the approximate optimal length for tension development in the gastrocnemia (Goslow et al., 1973). Twitch contractions of the muscle to be studied were evoked by stimulating the muscle at ten times threshold.

In a terminal experiment, muscle force was measured again but this time the contralateral control muscles (right MG in cat 1, right EDL and LG in cat 2) were also stimulated so that the force development in the control and experimental muscles could be compared. The stimulation of the experimental muscle was carried out through the implanted electrodes. The control muscles had dummy devices and for that reason they had to be stimulated using nerve cuffs implanted on the muscle nerve. The nerve cuffs were composed of a 1 cm piece of silastic tubing with a slit down its side, in which two stainless steel wires acted as bipolar electrodes. The force produced by each muscle was recorded using a force transducer which was attached to a clamp that held the distal tendon of the stimulated muscle in place. The limb was kept immobilized by drilling a hole in the tibia and passing a screw through the hole and subsequently securing the screw to the experimental surface.

Repair Procedures

On the fifth post-operative day, one of the animals, cat 1, was found to have removed the backpack and many of the wires were either damaged or ripped from their connections on the pin-connector. This animal was anesthetized using sodium pentobarbital and attempts were made to recover whatever leads could be located. To determine which lead came from the different electrodes, a series of stimulation trials were performed. We determined that the electrode in the left MG was still intact but the left TA electrode was damaged. Thus in this cat the experiment was reduced to an examination of the effects of stimulation and testosterone on the left MG only.

At several points throughout this experiment, the wire leads which were soldered to the pin-connector became detached. Each time that this occurred, the animal with the defective attachment was anesthetized with ketamine hydrochloride (9 mg/kg) and the attachment between the lead and the pin-connector was resoldered.

Blood Samples

During the experiment, blood samples were taken to measure levels of circulating testosterone, white blood cell count, red blood cell count and platelet counts. For each sample, 2.0 ml of blood was withdrawn from the jugular vein. The samples were assayed by a commercial testing laboratory (Clinical Laboratories, Kingston General Hospital) for total testosterone.

Muscle Stimulation

Stimulation of the implanted muscles was begun on the seventh day post-implantation. The animals were held gently with their forelimbs and chest supported slightly so that the animals bore part of their body weight on their hindlimbs. In this position the cats were reluctant to lift their hindlimbs from the supporting surface during stimulation. Trains of stimuli were delivered through the implanted electrodes to produce contractions. The amplitude of the pulse was determined on the basis of thresholds identified weekly for each individual muscle as well as the comfort level of the animal. Stimulation was initially administered at 2 times threshold and it was increased to a maximum of five times threshold.

Figure 6 shows the exact multiple of threshold used in each muscle. The stimulation level was increased to mimic a progressive resistance exercise program.

The muscles in the cat with two active electrodes were stimulated simultaneously such that the opposite actions of the two muscles contributed to the load on the muscle. In the animal with only one active electrode, the muscle was stimulated by itself and isometric contraction was ensured by applying resistance with the experimenter's hand.

A 3 second pulse train of 30 pps, increased to 50 pps after one week of stimulation, with a pulse width of 200 msecs was used. All stimulated muscles underwent four sets of eight contractions against resistance. The animals were exercised five days a week for five weeks. The duration of each contraction was three seconds and was followed by complete relaxation of the muscle for three seconds. The animal was allowed to rest for three to five minutes between sets. While the animal was stimulated, it was handled with affection, and given palatable food and water to keep it happy. Previous use of such stimulation suggested that the animals experience no apparent discomfort, but if any evidence of discomfort was observed, stimulation was stopped and comments were recorded.

Tissue Harvesting and Histology

At the conclusion of the study period, the animals were anesthetized so that force measurements could be taken for the final time as described above. Following these measurements, the experimental muscles were depleted of glycogen stores by stimulating at 50 pps for 300 msec every second, for a period of one hour at the same voltage that was used to stimulate the muscles during the final week of stimulation in the conscious animals. When this procedure was completed, the animal was given a lethal dose of sodium pentobarbital.

Incisions were made at all of the points where electrodes were implanted, being careful not to damage the muscles. All of the devices were removed carefully and inspected (figure 7). All 8 muscles from both cats were removed by careful dissection of the entire muscle including the tendon, from the origin to insertion. The individual muscles were blotted dry and weighed on a laboratory scale.

Each muscle was cut into a series of blocks about 2 cm in length in a defined rostrocaudal orientation from one end of the muscle to the other. Each block was mounted onto a numbered cryostat chuck using O.C.T. Compound and the orientation of each block was recorded. The blocks were coated with talcum powder and were immersed in liquid nitrogen and stored in liquid nitrogen until needed.

Several hours before sectioning the tissues, blocks were removed from the liquid nitrogen and allowed to warm in a cryostat at -20^0 C. Sections were mounted on slides subbed with chrome-alum gelatin and stained. A series of adjacent 16 mm transverse sections were cut from each muscle block for each of three stains. A standard frozen section staining protocol for hematoxylin and eosin (H&E) was used for analysis of muscle fiber size and number. For alkaline ATPase staining to determine fiber type, a modified procedure of Guth and Samaha (1970) was followed. The procedure described by Pearse (1961) was used for periodic acid-Schiff's (PAS) staining to assess the results of glycogen depletion.

Data Analysis

The slides obtained from the experimental muscles were the focus of the data for this study. Photomicrographs were taken of adjacent sections of muscle which had stained well with ATPase and PAS methods. The photographs were taken of areas with clear landmarks so that comparisons of the two differently stained sections could be carried out (figure 8). Using the PAS stained sections as a guide, the identity of substantially depleted fibers was noted on the matching ATPase sections. The ATPase sections were then analyzed using a computer-based optical imaging system (MCID-M2, Imaging Resources Inc.) which gives information regarding the optical density, which was subsequently used to classify fiber type, of individual fibers and also the cross-sectional area of marked fibers. These data were separated into 6 categories: type I, depleted; type I, non-depleted; type IIa, depleted; type IIa, non-depleted; type IIb, depleted; type IIb, non-depleted. The average cross-sectional areas and standard deviations of each fiber population was then calculated and the data was subjected to a two-tailed, unequal variance, t-test.

RESULTS

Thresholds and Muscle Stimulation

The electrical thresholds for stimulation of the implanted muscles were measured immediately following the implantation of the stimulating electrodes and were repeated on a weekly basis throughout the experimental period (figure 9, table 2). In cat 2 the threshold level for EDL dropped substantially from the first to second point of measurement at 4 days, whereas that of LG showed a modest increase at day 4. Thereafter, both muscles had relatively stable thresholds. However, in cat 1 the left MG demonstrated a sharp increase of more than 10 V in its threshold level during the last half of the experiment.

Stimulation of the implanted muscles was initiated on the seventh day post implantation using pulses of 1.4 to 2.1 times threshold levels. The amplitude of the stimulation pulses were increased as the animals grew more accustomed to the procedure as summarized in figure 10. The levels of stimulation were adjusted on the basis of the apparent comfort level of the cat, thus there appear to be jumps in the strength of stimulation from time to time. The extremely high level of stimulation used on left MG (cat 1) was due to the high threshold level observed for that muscle (figure 9). The frequency used to stimulate the muscles in both cats was increased from 30 Hz to 50 Hz during the second week of stimulation.

As each muscle was stimulated, the contracting muscle was palpated by the experimenter to ensure that a forceful contraction was occurring. Although initial protocols had dictated a gradual increase in stimulus strength to 10 times threshold, cats appeared to be concerned by the very strong contractions elicited as stimulation was raised over about 4 times threshold. At such levels, the cats began to resist restraint. Thus only stimulus levels that were well tolerated by the cat were adopted. On the few occasions that no contraction was felt, it was found that the connection between the electrodes and the 6-pin connector had become detached. When this occurred, the wiring was repaired (see Methods, Repair Procedures).

Testosterone Levels

The results of the weekly blood assays are displayed in figure 11 and table 3. From this summary it is evident that the circulating levels of total testosterone in both cats was elevated by 5000 and 3000 % from normal levels previously measured in cats 1 and 2 respectively. After peaking at about day 11, the levels progressively dropped until only low values were present.

During the course of the experiment the behavior of the animals was observed to change from their pre-implantation days. Sexually aggressive behavior of these previously docile ovariectomized female cats was observed, and they were moved to a separate room to limit their advances to other females in the unit in which they had been housed. As the experiment progressed however, this behavior subsided and the animals seemed to return to their normal patterns of activity.

Force Measurements

The force of the contractions elicited by the stimulating electrodes was measured weekly using a cast fitted with a force transducer. The cats tolerated the cast only if the cast was fitted quite loosely. As a result, this method of measuring force proved to yield inconsistent results from day to day. It was therefore decided that the results would prove to be of little use and they were excluded from further analysis.

The terminal experiment of measuring the force of both the experimental and the control muscle did not use the specially designed cast, but instead encountered other methodological problems. The method of recruitment of the muscle fibers was different between the control and experimental muscles. In the former, stimulation of the whole muscle nerve was applied at supramaximal strengths and thus recruited the entire muscle. In the latter, stimulation was administered using the implanted device and stimulus strengths were limited to those comparable to levels used in alert cats in order to avoid

compromising glycogen depletion. In all three muscles, stimulation using the device caused a less forceful contraction than that elicited by supramaximal stimulation of the contralateral muscle.

Muscle Weight

Muscle weights appeared to differ only slightly when stimulated and non-stimulated muscles were compared. A summary of the weights of all implanted muscles are compared to contralateral control muscles in figure 12. The stimulated left MG (cat 1) was found to weigh 10.89 g whereas the contralateral control muscle weighed 9.03 g. The non-stimulated MG muscles from cat 2 had weights of 12.30 (left) and 12.22 g (right). In both animals the weights of the left muscles were somewhat larger but the changes are not sufficient to confirm the presence of hypertrophy in response to stimulation.

The data from the muscles of cat 2 also yielded only small differences in weight. The stimulated left LG muscle (21.88g) was slightly larger than the contralateral control muscle (20.96g, 4% difference) but the stimulated EDL muscle (3.56g) weighed slightly less (4 %) than its contralateral control (3.70g). Thus, it was not possible to recognize a significant difference in either case.

Glycogen Depletion

On the last day of the experiment, the muscles implanted with active electrodes were subjected to a stimulation protocol that was intended to deplete their glycogen stores. These muscles continued to contract for the duration of the stimulation procedure but the contractions appeared to weaken slightly as the procedure progressed. To assess the results of the glycogen depletion, sections from the depleted muscles were stained for glycogen content. When the sections were viewed under a microscope, some fibers were blanched indicating that they had been depleted of their glycogen stores. Other fibers appeared to have lighter staining profiles than was typical for fibers in adjacent, non-stimulated muscle or control muscle sections, but were not fully blanched. Fibers from experimental muscles that were stained very intensely (figure 13), like fibers in control muscle sections, were presumed to be unstimulated. Fibers showing a significantly or fully blanched appearance were considered to be depleted and thus stimulated.

Reconstructions of two of the experimental muscles were created with the aid of a microfiche reader and a light microscope (figures 14 and 15). Sections of muscle tissue were divided into areas of varying levels of depletion based on the relative proportions of blanched profiles. From these figures it is evident that the areas of depletion were relatively small and were depleted more thoroughly in regions around the stimulating electrode.

Cross-sectional Areas of Depleted Fibers

In most PAS-stained sections from stimulated muscles, only the smaller fibers in the section usually appeared to be depleted. These fibers were commonly found in the middle of the fascicles rather than on the periphery. Figure 13 shows an example of a typical photomicrograph that was obtained from a section of a stimulated muscle.

The mean cross-sectional areas of randomly selected fibers in ATPase stained sections of three muscles are displayed in figure 16. This figure shows the mean cross-sectional areas of the fibers in the non-stimulated right EDL (cat 2), the stimulated left EDL (cat 2) and the stimulated left MG (cat 1). Evaluations of fibers in LG were complicated by a relatively indistinct pattern of blanching that made it difficult to select some depleted fibers with assurance. The differences in cross-sectional area between the stimulated and the control EDL muscle was found to be statistically insignificant. However, there was a statistically significant difference in the mean cross-sectional areas of the fibers in EDL muscles when compared to the fibers of the stimulated MG.

TYPE IIB FIBERS

Stimulated muscles contained a mix of depleted and non-depleted fibers of different types. Thus the cross-sectional areas of depleted and non-depleted fibers of different fiber types were investigated more

fully in two stimulated muscles, EDL and MG, as well as one control muscle, EDL. Figure 17 summarizes the mean cross sectional areas of the type IIb (fast fatiguable, glycolytic) fibers and compares the cross-sectional areas of the non-depleted fibers to those of the depleted fibers in the only muscle in which depleted fibers were found (L-EDL, cat 2). The cross-sectional areas of depleted fibers were not significantly different from the non-depleted fibers of this muscle ($p>0.3$). The non-stimulated muscle had significantly larger type IIb fibers than the stimulated muscle ($p<0.05$). The non-depleted IIb fibers in the stimulated left MG (cat 1) were found to be significantly larger ($p<0.05$) than those in the stimulated left EDL (cat 2) but there were no significant differences with respect to the fibers from the non-stimulated right EDL of cat 2 ($p>0.1$).

TYPE I FIBERS

Type I (slow oxidative) fibers from each of the three muscles were compared and the results are summarized in figure 18. In the stimulated left EDL (cat 2), the mean cross-sectional areas of the depleted type I fibers were significantly larger than the those of the non-depleted fibers ($p<0.001$). However, the type I fibers from the contralateral unstimulated right EDL had a mean cross sectional area that was also significantly larger than the non-depleted type I fibers in the stimulated muscle ($p<0.005$). All of the type I fibers found in the section examined from the stimulated left MG (cat 1) were depleted and thus could not be compared to non-depleted fibers in the same muscle section to determine if there were any differences in cross sectional area.

TYPE IIA FIBERS

The best analysis that could be performed was that of the cross-sectional areas of the type IIa fibers because there was an abundance of both depleted and non-depleted fibers of this type in both of the experimental muscles examined. Figure 19 shows that there were no significant differences in cross-sectional areas of the non-depleted fibers across the control muscle and the two experimental muscles ($p>0.3$). What was striking was that the mean cross-sectional area of the depleted IIa fibers in both the left EDL and the left MG were significantly larger than the non-depleted fibers in each respective muscle ($p<0.05$ in both cases).

To summarize the results of the comparison of the depleted fibers to the non-depleted fibers we have seen that depleted type IIb fibers were not significantly different in size from non-depleted fibers, that depleted type I fibers were larger than non-depleted fibers in the one muscle that permitted these comparisons, and that depleted type IIa fibers were significantly larger than non-depleted fibers in both muscles that permitted this comparison.

DISCUSSION

This feasibility study was undertaken to determine whether a combined regimen of local testosterone administration and electrical stimulation was able to cause hypertrophy of feline hindlimb skeletal muscles. This experiment was successful as it identified two areas where difficulties with the methods are present. First, it was not possible to ensure that high doses of testosterone could be localized to a single set of target muscles. Because systemic levels of testosterone were greatly increased by the 'local' pellets, the initial goal of this study was no longer achievable. Thus, the protocol was modified to determine the effects of electrical stimulation on hindlimb muscles of testosterone exposed cats.

Second, several technical difficulties were encountered. Electrode lead breakage and/or damage was experienced, which in some cases was repairable. However, we suspect that some form of electrode change may have contributed to the unusual change of threshold in cat 1 midway through the experiment. In addition, it was difficult to ensure a consistent stimulation level. Because cats reacted to high stimulation levels, it was necessary to constrain the stimulus strength to a level later shown to recruit only a portion of the muscle. This problem may have been responsible for the failure to see changes in muscle weight.

However, the present experiments did have an interesting and unexpected result. It appeared that the majority of the fibers that were recruited by intramuscular stimulation were type I and type IIa fibers, as opposed to type IIb fibers that are generally recruited first by stimulation of the peripheral nerve. The stimulated type I and IIa fibers showed significant increases in cross-sectional areas when compared to non-depleted fibers of the same type within the same muscle. These results suggest that the stimulation was effective in causing hypertrophy of at least some of the stimulated fibers.

Methodological Problems with Testosterone

The local testosterone pellets used in this study were advertised to supply the target tissue with a high local concentration of testosterone with no significant amount (less than 5%) reaching the systemic circulation (personal communication, Dr. Schafie, Innovative Research of America). However, within the first week we discovered that the claims made by the manufacturer were erroneous and that the 'local' pellets were releasing testosterone into the general circulation at a rate sufficient to elevate blood levels of the hormone up to 50 times the normal level of testosterone in spayed female cats. Therefore, we have concluded that these pellets are not useful for local administration of testosterone. It is possible that although the systemic levels of testosterone were elevated, there may have been greater levels of the androgen at the site of the target muscle. To determine if this is indeed the case, testosterone assays will be carried out on muscle tissue extracted from the left and right muscles.

In previous studies involving testosterone, systemically administered testosterone has been used. Kuhn and Max (1985) implanted crystalline testosterone propionate filled Silastic tubing to administer 2.5 mg of androgen per day . This treatment resulted in a 20 fold increase in serum testosterone levels. In our study, the pellets implanted contained a total of 20 mg that was supposed to be released at a constant rate over a period of 60 days. However, we saw a sharp rise and fall in circulating levels of androgen, pointing to a high release rate during the first week of the experiment. In the experiment of Kuhn and Max, the release rate was such that 20 mg was released in 8 days. This release rate is consistent with our results and suggests that the pellets used presently had a release rate of about 2.5 mg/day.

The high testosterone levels present in the ovariectomized animals likely had effects on many of their tissues. Joubert et al. (1995) measured the incorporation of [^{3}H] thymidine into satellite cells of the levator ani muscle of female rats to determine if they increase DNA synthesis when exposed to testosterone. They found that testosterone induces satellite cell proliferation. However, the levator ani muscle is sexually dimorphic and is rarely affected in females because of relatively low levels of circulating testosterone. Levator ani is androgen sensitive, however, and thus is responsive to testosterone administration. Whether these results can be extrapolated to the present study is debatable because the hindlimb muscles of the cat are known to be relatively insensitive to androgens. Examination of the comparative response of sexually dimorphic muscles of the cats to testosterone may have provided interesting results.

It was noted that the spayed female cats studied here had increased levels of aggressiveness and displayed characteristics of estrus despite the fact that they had been ovariectomized. It is presumed that this behavior was the result of the increased levels of testosterone in the body. However, the behavioral observations were not made by naive blinded observers but were reported by individuals with knowledge of the type of treatment the animals were undergoing.

Any future experiments that attempt to administer testosterone locally will have to be planned carefully. A study examining local androgen effects on rat muscle (Rand et al., 1992) used an administration method similar to the pellets we used with greater success. Rand et al. made a reservoir from Tygon tubing, filled the reservoir with crystalline testosterone, sealed the reservoir with Silastic and sutured the entire apparatus to the muscle being studied. Their preparation was successful in localizing the effects of the hormone to the target muscle and suggests possible future directions for this type of study.

Changes in Stimulus Thresholds

During the weeks after surgery, only one device displayed threshold changes that typify implanted stimulators whose stimulus parameters have been measured over time (Cameron et al, 1997). In many instances, such as that of the EDL stimulation reported here, a rapid decrease in stimulation threshold of EDL (cat 2) was observed within the first week after implantation. This could be explained by the normal immune response to an implanted foreign object. The implantation of a device results in inflammation and the formation of a protective barrier between the device and the host tissue. This foreign body reaction is consistently observed following implantation of even biocompatible devices. The reaction usually stabilizes over a period of time and results in a fibrous capsule forming around the implant. The acute inflammation present following surgery subsides, although a low level persists at the surface of the implant for the duration of the implantation. It is likely that the area surrounding the implant was full of fluid immediately following implantation and as the response subsided, the fluid in the area was absorbed. The distance from the device to underlying excitable nerve branches would have decreased as a result, thus reducing threshold levels for muscle stimulation.

This reasoning fails to explain the observation that the same threshold changes were not seen in all implanted muscles. The LG (cat 2) had a relatively consistent threshold and that of the MG (cat 1) showed a dramatic increase in threshold. The large increase observed may have been due to a poor solder connection between the lead from the electrode and the pin connector. Repeated repairs were required to fix the connections between the wires and the backpack. As the quality of the connection degraded, the stimulus intensity may have had to increase in order for the same level of muscle activation to occur.

An alternative explanation for the changes seen in the threshold level can be associated with the movement of the electrode within the muscle. The electrodes were attached to long leads on which traction could occasionally be placed when the cat groomed or pulled on its backpack. If the electrode moved away from the nearby motor axon, the currents to which the axons were exposed would have been reduced resulting in an increase in the strength of stimulation required to elicit a contraction. Conversely, movement of the electrode towards the motor axons would increase the amount of current impinging on the axons resulting in a decrease in the threshold level.

The problems discussed here are typical of stimulation methods normally used for animal experimentation. Percutaneous electrodes have been regarded as problematic because wires have to be lead through the skin where they can be chewed or damaged, and because the fine wires used are susceptible to breakage. For these reasons an alternative method of stimulation will be used for any future studies involving muscle. The fully implantable microstimulator currently under development with Advanced Bionics (BION™) will alleviate some of the problems encountered in this study. This device does not require percutaneous wires and thus can stimulate muscles without the complications of lead breakage. Further, the design of this device hinders migration (Fitzpatrick et al., 1997) so that movement of the electrode within the muscle is unlikely to occur and to cause a change in threshold. The lack of change in threshold level of these devices has been previously documented (Cameron et al., 1997)

Evaluation of Muscle Parameters

FORCE MEASUREMENTS

The force measurements made weekly on the conscious animals and the ones performed during the surgery at the end of the experiment were not found to be useful for assessing changes in muscle force production over the testing period. We assume that the large variations observed in forces measured using the instrumented cast arose because it was difficult to fit the cast reproducibly in highly mobile animals. Further, it was impossible to ensure that the same angle of the joint was used in each trial. These problems led us to exclude measurements of force from the alert animals from further analysis. In future studies undertaken to examine the force-generating capabilities of muscles in the conscious animal, a more rigorous and standardized procedure is needed. From the difficulties encountered in this

experiment, it has become apparent that measuring these values in the conscious animal may be impossible and that it may be necessary to anesthetize the animals for the procedure.

The final set of force measurements made on the unconscious animals involved a comparison of the force generated by the implanted muscles and the control muscles. Contractions of the experimental muscles were carried out through the implanted devices whereas nerve cuffs were used to stimulate the control muscles. The nerve cuff electrodes depolarize all of the axons that innervate the muscle, whereas the implanted electrode only depolarizes certain branches of the nerve. This differential recruitment was evident in the glycogen depletion patterns observed in the experimental muscles (see below). Thus, the results of these final measurements were not appropriate to evaluate the potential presence of muscle strengthening. If we were not interested in the extent of muscle recruitment, we could have obtained a comparison of whole muscle force in the experimental as well as the control muscle by placing a nerve cuff electrode on the parent bundle nerve. However, we were concerned that such stimulation would deplete nerve fibers not recruited by the intramuscular device, thereby confounding the results of subsequent glycogen depletion.

Changes in Muscle Mass

The level of stimulation used in this experiment resulted in significant contractions of the muscles, but there was no definitive difference in the weight of the stimulated muscle in comparison with the non-stimulated control on the contralateral side. A marked increase in muscle mass of the stimulated muscle had been expected on the basis of literature that has reported hypertrophy following stimulation. This whole muscle hypertrophy has been attributed either to individual fiber hypertrophy, fiber hyperplasia, or a combination of the two. However, histological findings of this study showed that only a minority of fibers appeared to be activated in each of the stimulated muscles. It is possible that slight hypertrophy could occur that would be below the level of sensitivity of the weighing method used.

Hypertrophy of Muscles By Hormonal Manipulation

Although the serum testosterone levels were elevated, it was predicted that the level in the leg implanted with a pellet would have greater levels of testosterone than the control leg. This prediction cannot be validated until biochemical assays on the muscles are carried out. Such testing has yet to be undertaken. Nevertheless, many studies have shown that testosterone administration alone can cause an increase in muscle size. For example, the study by Bhasin et al. (1996) showed that administration of supraphysiological doses of testosterone to normal men who did not partake in any exercise significantly caused an increase in the areas of their triceps and quadriceps muscles. Dorlochter et al. (1994) demonstrated that sexually dimorphic muscles of castrated male frogs hypertrophied within 7 days of implantation of testosterone implants. The increase disappeared a week later only to be followed by a further increase in muscle size.

Not all of the studies examining the effects of testosterone on muscle size have reported muscle hypertrophy, however. Kuhn (1985) showed that chronic testosterone treatment did not cause muscle hypertrophy in female rats. In that study a tenotomy model was used to overload the muscles being studied and testosterone propionate was administered with subcutaneous capsules. No significant differences were found when the muscle weight of testosterone treated animals was compared to similarly tenotomized animals.

Hypertrophy of Muscles By Increasing Workload

The failure to demonstrate changes in muscle weight in the present study is not consistent with a number of previous studies in which compensatory muscle hypertrophy was seen to follow increases in muscle work. Goldberg (1975) showed that increasing the work load of a muscle by tenotomizing a synergistic muscle leads to an increase of the intact muscle's weight by 30 to 50 %. However, it is difficult to relate the results of Goldberg to this study for two reasons. First, the studies by Goldberg were carried out on the rat soleus muscle which is a predominantly slow twitch muscle. The muscles under

study in our experiment were predominantly fast, mixed muscles, as determined by the ATPase staining characteristics reported here and in a previous study (Ariano et al, 1973). Also, following tenotomy, the work load on a muscle was increased continuously. The type of stimulation employed in the present study caused the muscle to do high-intensity work for a short period of time. Thus different stimulation paradigms may have to be used in order to observe the increases observed by Goldberg.

A study by Giddings et al. (1985) employed a protocol whose effect on muscle behavior resembled more closely the type of work induced by brief stimulus trains in this study. Cats were trained to lift weights voluntarily with their forelimb for a food reward. Although not specified in the experimental report, it is assumed that the weight lifting exercises were short in duration. Giddings et al. (1985) found a 23.6 % increase in weight of the exercised muscle compared to the contralateral control and a 48.6 % increase in weight when the exercised muscle was compared to those of control animals. The muscles that had been exercised were found to demonstrate degenerative changes as well as hypertrophied fibers. Increased percentages of unusually small fibers in addition to an increased percentage of unusually large fibers were also observed. The small fibers were considered to be regenerating fibers because they demonstrated no degenerative characteristics.

Our study differed from that of Giddings in that the work induced by the intramuscular devices was not voluntary. Because our study involved the application of electrical stimulation to muscles, it was considered ethically improper to increase the stimulation level to one which would have produced very strong contractions, discomfort, and possible pain. The use of electrical stimulation to induce contractions of muscles whose force exceeds their maximal voluntary capabilities has been described previously in human experiments (Delitto et al., 1989). However, humans are better able to tolerate neuromuscular electrical stimulation. A person who appreciates the goal of the experiment will be much more willing to undergo intense stimulation than an animal whose understanding is limited to the anticipation of a food reward given after the stimulation.

The experiment that relates most closely to the study presented here was conducted by Inoue et al. (1993). They showed that electrical stimulation of the rat gastrocnemius resulted in a modest 8.3 % increase in muscle mass after 27 days of stimulation, as compared to controls. However, Inoue et al. anesthetized their animals before delivering large amplitude stimuli (10 V, 2 ms duration) through needle electrodes inserted acutely into the muscles. Three sets of 10 2-second contractions at 100 Hz, separated by 5-second rest periods, were performed daily. Although it is difficult to compare stimulation protocols using different electrodes and species, we might expect that the stimuli delivered by Inoue et al. (1993) were much stronger and more efficacious in producing whole muscle contractions. Our stimulation protocol used pulse durations of only 0.2 msec and 50 Hz frequency. Voltages in the same range as those used by Inoue et al. (1993) were employed for only one muscle with a high threshold level. Further, cat muscles are much larger than comparable rat muscles, lending to easier maximal recruitment from a single stimulus site. Thus any future studies attempting to induce hypertrophy of muscles using electrical stimulation must consider the high levels of stimulation that appear to be required for any changes in muscle weight to be seen. However, we must keep in mind the welfare of the experimental subjects being subjected to electrical stimulation.

An important functional question is the usefulness of present results for the alternate goal of developing a stimulating regime that would reverse the muscle atrophy seen in patients with disuse atrophy due to damage of spinal or descending pathways. Unlike human patients, the hindlimbs of the cats used in this study were very active and the cats used were healthy. It is possible that these muscles are already near-optimal levels of condition because they are used frequently during jumping and locomotion as the cats ranged freely in their housing units. Attempts to induce hypertrophic changes in these muscles may be ineffectual due to the already healthy condition of the muscle.

A better model of the condition that we seek to remediate may be achieved by immobilizing the muscles under study. Casting both limbs to prevent movement and then applying stimulation to only one would offer a better comparison of the effects of stimulation in preventing muscle atrophy. Casting protocols have previously been developed in rodents (Maier et al., 1976). Tail suspension in rodents is a commonly used approach that prevents the use of the hindlimb muscles and would also permit an analysis of the effects of stimulation on muscles of off-loaded limbs. While both of these methods are feasible, they are not completely adequate because voluntary contractions of the muscles can still be performed and thus the model differs from the clinical condition that we are attempting to remedy.

Spinalizing the animals would be a more appropriate method of replicating the clinical condition, however, spinalization also has drawbacks. The amount of care required for such animals is great and results can be complicated by the residual function available by way of reflex pathways. The level of reflexive muscle activation may vary from animal to animal in a way that is different from human patients and may contribute an undesirable level of variability to the results.

The best model may involve the use of tetrodotoxin. Tetrodotoxin releasing cuffs placed chronically on the muscle nerve have been shown to prevent the transmission of centrally originating commands to the muscle by blocking sodium channels on the nerve. This method leaves the innervation of the muscle intact, as in spinal cord injuries, but voluntary efforts to activate the muscles would not be effective. Thus, the muscle would atrophy thereby providing an ideal model for this type of study. The experimental muscle could then be subjected to a stimulation protocol similar to that which we have used, or to other protocols which might have differential effects.

Glycogen Depletion

The stimulation protocol used in this study did not cause any large changes in muscle mass. This finding could be attributed to the relatively low levels of stimulation that appeared to recruit only a minority of fibers, as evaluated by the results of glycogen depletion. It was believed that our stimuli recruited most of the muscle because the contractions elicited were very strong, sometime necessitating reduction of the stimulus for fear of harming or disturbing the animals. However, if only a small part of the muscle was in fact active, it is not surprising that there was little or no change in whole-muscle mass. For example, a 10 % increase in weight of stimulated fibers would appear as only a 1 % increase in whole muscle mass if 10 % of the fibers were stimulated. Thus, an evaluation of hypertrophy would have to be directed at the stimulated fibers themselves.

An especially interesting finding of the glycogen depletion was that most of the depleted fibers were types I and IIa. The observation is contrary to the result that is generally expected following electrical stimulation of a muscle nerve, where the large diameter motor axons that innervate the type II fibers are more easily excited by electrical stimulation than the small size axons that innervate the type I fibers (Kim et al., 1995). This pattern of large-axon preference is in the reverse of the recruitment pattern occurring physiologically, in which the type I motoneurons are recruited first during a voluntary muscle contraction.

The reversal of recruitment order typically seen using nerve cuffs has posed a problem for the use of FNS to rehabilitate disabled patients. Because the nerve cuff electrodes first recruit the fast-twitch, glycolytic type IIb fibers that are innervated by the large diameter motoneurons, the contractions occur in fibers unsuitable for long-duration activities. Thus, patient's muscles quickly become fatigued during stimulation sessions because of rapid depletion of glycogen stores by the type IIb fibers. The current study has shown that the more fatigue resistant type I (slow, oxidative) and type IIa (fast fatigue-resistant, oxidative) fibers can be recruited by FNS at least as efficaciously as IIb fibers using intramuscular electrodes. This result is clinically very important. Replication of these observations in human subjects would support the use of intramuscular stimulation for FNS applications.

The problem of false-positive and false-negative staining must be addressed when considering the results of glycogen depletion. Glycogen depletion results were analyzed on muscles stimulated via the implanted electrodes for over one hour. Muscle sections were then stained with PAS to assess which fibers were recruited during our stimulation procedures. However, the results of the glycogen depletion reflect only the fibers that were recruited during the terminal experiment. Despite identical stimulation parameters as the chronic muscle exercise, it is possible that the fibers that were stimulated during that terminal experiment were not wholly representative of those that had been stimulated for the previous 5 weeks. Small shifts in the position of the electrode during terminal dissections may have slightly altered the physical relationships between the nerves and the electrode.

PAS sections can be difficult to interpret because the stain shows the glycogen content of the muscle cells, which assumes the substantial presence of glycogen in all fibers before the depletion protocol is employed. Analysis of control muscles also stained by this method (results not presented) indicated that

the type I fibers, which have a lower glycogen content because of their metabolic characteristics, appear to stain more lightly than other fibers in the section, even when not stimulated. This observation raises the possibility that the fibers which were considered as depleted were simply displaying a profile normal for slow fibers. To avoid counting a large number of false-positive fibers, we designated a fiber as depleted only if it was unambiguously blanched. Fibers which may have been depleted according to their partial staining were designated as non-depleted. As a result, the number of depleted fibers may have been under-estimated.

Increases in Fiber Cross-sectional Area

The results of this study show that glycogen-depleted fibers can show significant increases in cross-sectional size. In the stimulated EDL muscle, there were significant increases in the size of the depleted types I and IIa fibers as compared to non-depleted fibers in the same muscle. Similarly, type IIa fibers in the MG that were thought to be stimulated had greater cross-sectional areas than non-stimulated type IIa fibers.

Previous studies examining the effects of electrical stimulation on the size of individual muscle fibers have shown mixed results. Cabric et al. (1987) subjected human males to electrical stimulation at a frequency of 50 Hz for a period of 21 days and observed significant increases in fiber size in comparison to controls. These findings corroborate the observations made in the present study that used a similar stimulation protocol to produce muscle hypertrophy.

The use of different protocols results in different patterns of muscle-fiber change. Greve et al. (1993) administered 3 ms duration pulses at a frequency of 20 to 30 Hz for 5 seconds and showed that no significant changes occurred in the diameters of the fibers, although a significant increase in the number of type IIa fibers was seen. This study involved human subjects whose spinal cords were lesioned at least a year before the study. Thus, the long-time disuse atrophy experienced by the subjects may have been difficult to reverse. Although changes in the fiber numbers have not yet been analyzed in the present study, it is conceivable that both the size and number of type IIa fibers increased in response to stimulation. Such a finding would be promising for the rehabilitation of spinal cord injured patients.

CONCLUSION

It was evident from the results presented in this report that the so-called 'local' release testosterone pellets did not function according to specification. Another method of locally releasing testosterone in muscles must be designed before continuing this series of experiments. However, the electrical stimulation protocol and the majority of the methods used to measure muscular changes induced appeared to be feasible for the study of muscle responses to chronic stimulation. The stimulation protocol used in this study was effective in recruiting type I and IIa fibers together with or even in preference to type IIb fibers. This finding has important clinical implications because it suggests that the common problem of fatigue experienced by patients undergoing FNS treatment using muscle-nerve stimulation may be reduced by using an intramuscular stimulation method. Further study also confirmed that hypertrophy can be produced in stimulated fibers with relatively modest stimulation strengths and relatively short stimulation periods. However, results of this study must be interpreted cautiously. Because this was a feasibility study, all findings reported here are preliminary and a larger sample of animals would be needed to make conclusions with any confidence. To better understand the usefulness of these protocols to remediate muscle atrophy, further studies on animal models that more closely resemble the clinical condition of patients need to be carried out.

PREVIOUS ACHIEVEMENTS IN THE LABORATORY

As part of the summer work experience program (SWEP), I spent the summer as an employee in Dr. Richmond's laboratory. During my time as a summer student, I was able to familiarize myself with the workings of the laboratory, Animal Care Services, and some general research that was taking place under her supervision. I performed several sessions of animal training (not with the animals used in this

research project), some histology for other studies in the lab and some minor animal surgeries. I also learned about the functioning of the implantable stimulator that is currently being developed. However, no experiments were performed and no data was collected prior to my registration in the course. The advantages I gained from spending the summer in Dr. Richmond's lab was a knowledge of the laboratory organization and some general methods used in research.

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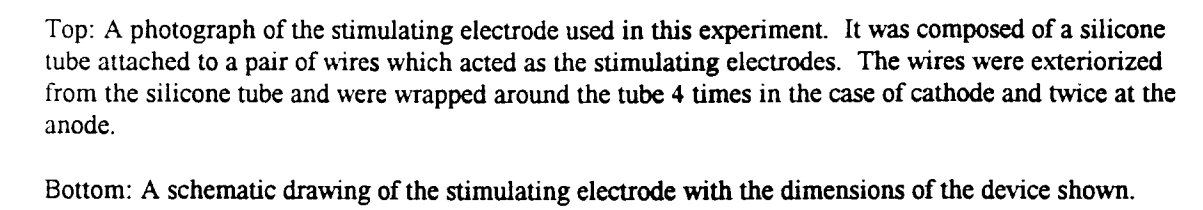
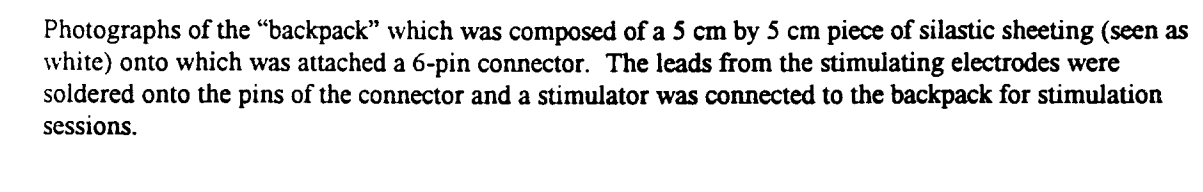
FIGURE 1. STIMULATING ELECTRODES



Top: A photograph of the stimulating electrode used in this experiment. It was composed of a silicone tube attached to a pair of wires which acted as the stimulating electrodes. The wires were exteriorized from the silicone tube and were wrapped around the tube 4 times in the case of cathode and twice at the anode.

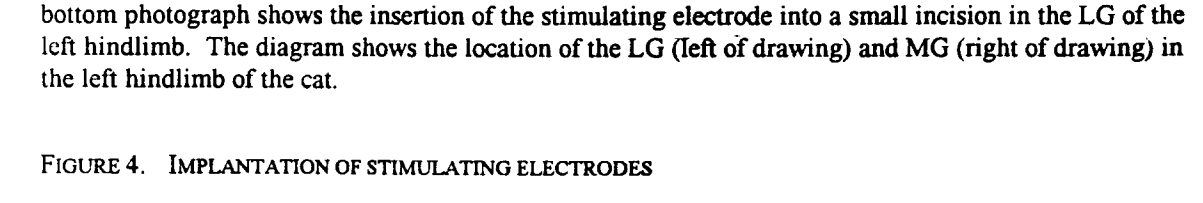
Bottom: A schematic drawing of the stimulating electrode with the dimensions of the device shown.

FIGURE 2. BACKPACK



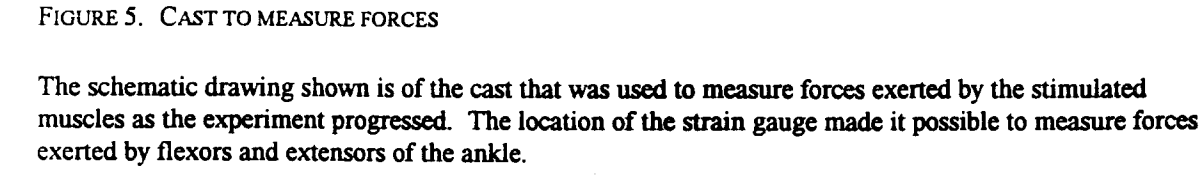
Photographs of the "backpack" which was composed of a 5 cm by 5 cm piece of silastic sheeting (seen as white) onto which was attached a 6-pin connector. The leads from the stimulating electrodes were soldered onto the pins of the connector and a stimulator was connected to the backpack for stimulation sessions.

FIGURE 3. IMPLANTATION OF STIMULATING ELECTRODES



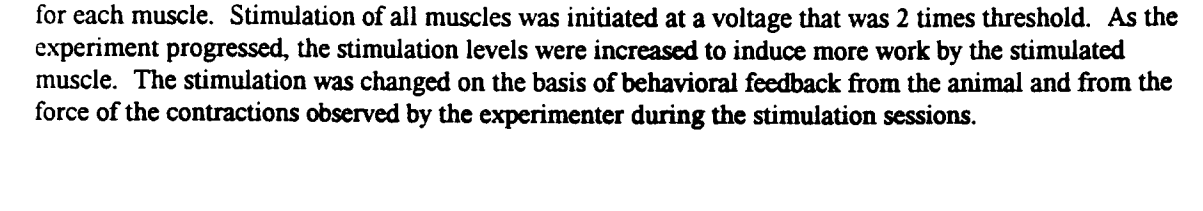
These photographs and the drawing demonstrate the locations of the implanted materials in this experiment. The top photograph is of the testosterone pellet in a piece of silastic tubing which was sutured to the connective tissue between MG and LG at the location indicated by the top arrow. The bottom photograph shows the insertion of the stimulating electrode into a small incision in the LG of the left hindlimb. The diagram shows the location of the LG (left of drawing) and MG (right of drawing) in the left hindlimb of the cat.

FIGURE 4. IMPLANTATION OF STIMULATING ELECTRODES



The photograph and line drawing demonstrate the location of the stimulating electrode in the EDL. The photograph to the left shows where the electrode will be situated once it has been inserted into the muscle. The drawing on the right shows the location of the electrode in terms of the whole leg.

FIGURE 5. CAST TO MEASURE FORCES



The schematic drawing shown is of the cast that was used to measure forces exerted by the stimulated muscles as the experiment progressed. The location of the strain gauge made it possible to measure forces exerted by flexors and extensors of the ankle.

FIGURE 6. STIMULATION LEVELS WITH RESPECT TO THRESHOLD

The line graph shown represents the stimulation levels that were used in terms of the thresholds measured for each muscle. Stimulation of all muscles was initiated at a voltage that was 2 times threshold. As the experiment progressed, the stimulation levels were increased to induce more work by the stimulated muscle. The stimulation was changed on the basis of behavioral feedback from the animal and from the force of the contractions observed by the experimenter during the stimulation sessions.

FIGURE 7. ELECTRODES UPON REMOVAL FROM THE MUSCLE

Top: This photograph of the EDL shows the condition of the stimulating electrode at the end of the experiment after it had been removed from the muscle. As can be seen, the electrode was undamaged and did not cause a noticeable amount of damage to the muscle.

Bottom: This photograph of the LG also demonstrates the lack of damage to the electrode or the muscle following five weeks of stimulation.

FIGURE 8. ADJACENT SECTIONS OF MUSCLE STAINED WITH ATPASE AND PAS

Top: The photomicrograph shows a well stained ATPase section. The very dark muscle fibers were identified as type IIb fibers. The intermediately stained sections were classified as type IIa fibers. The white fibers in the top photomicrograph were determined to be type I fibers. Those fibers marked with a star are those that were determined to be depleted of their glycogen content.

Bottom: The photomicrograph displayed is of a PAS stained section of muscle that was adjacent to the ATPase stained section that is shown in the top photomicrograph. The dark red fibers are those that contain glycogen. The pink fibers are those that may be partially depleted of glycogen but were not considered to be depleted. The white fibers marked with stars in the bottom photomicrograph were classified as depleted and their fiber type was subsequently determined on the basis of their ATPase staining characteristics.

FIGURE 9. THRESHOLD LEVELS

The line graph shows the threshold levels measured weekly for each of the stimulated muscles. The thresholds for LG and EDL were relatively consistent throughout the experimental period. MG showed a large increase in threshold during the last half of the experiment.

FIGURE 10. STIMULATION LEVELS

The levels of stimulation used for each muscle were plotted as a function of the day of the experiment. The stimulation level was very high for MG because of the high threshold levels it displayed. The stimulation levels of the other two muscles were adjusted on the basis of their thresholds.

FIGURE 11. TESTOSTERONE LEVELS

The level of serum testosterone as measured from weekly blood samples. The levels of circulating testosterone were elevated well above normal levels for spayed female cats. The serum testosterone levels began to decline 11 days after implantation.

FIGURE 12. MUSCLE WEIGHTS

The bar graphs represent the weight of the 8 muscles dissected from each cat after 5 weeks of stimulation. The dark bars show the weight of the unstimulated muscles from left hindlimbs. The white bars show the weights of the unstimulated muscles from right hindlimbs. The shaded bars show the weights of the stimulated muscles from the left hindlimbs. The left legs were also implanted with testosterone pellets. There were no significant differences in weight between the stimulated muscles and the unstimulated muscles.

FIGURE 13. MUSCLE SECTION STAINED FOR GLYCOGEN CONTENT

The photomicrograph of a muscle section stained for glycogen content shows fibers that are clearly depleted of glycogen and those that are not. The fiber with the black star is an example of a fiber that is fully depleted of glycogen. These depleted fibers were predominantly found in the middle of the fascicle rather than on the periphery. Those fibers marked with white stars are examples of fibers that were not considered to be depleted of glycogen.

FIGURE 14. RECONSTRUCTION OF THE STIMULATED MG

The drawing shown is a reconstruction of the stimulated MG based on the percentage of depleted fibers in areas of the muscle tissue. What is evident from this drawing is that the amount of depletion in this muscle was very small. This may be interpreted as meaning that very few fibers in the muscle were recruited during the stimulation sessions.

FIGURE 15. RECONSTRUCTION OF THE STIMULATED EDL

The drawing shown is a reconstruction of the stimulated EDL based on the percentage of depleted fibers in areas of the muscle tissue. What is evident from this drawing is that there were some areas of the muscle that were well depleted, but they were very few and far between. the interpretation of this result may be that the stimulation sessions failed to recruit a significant number of fibers.

FIGURE 16. MEAN CROSS-SECTIONAL AREAS OF ALL FIBER TYPES IN THREE MUSCLES

The mean cross-sectional area (CSA) of all fiber types from three muscles is plotted in the bar graph. An unstimulated right EDL, the stimulated left EDL and the stimulated left MG were used. The differences in CSA between the two EDL muscles is not significant.

FIGURE 17. CROSS-SECTIONAL AREAS OF TYPE IIB FIBERS

The mean CSA of depleted type IIb fibers and undepleted type IIb fibers is plotted in the bar graph. Depleted fibers, represented by the white bars, of this type were found only in the stimulated EDL. The

depleted fibers from the stimulated EDL were smaller than the undepleted fibers (dark bars) from the same muscle, but the difference was not significant.

TABLE 1. LOCATION OF STIMULATING AND DUMMY ELECTRODES

This is a summary of the locations of stimulating and dummy electrodes in the two cats used in this experiment. Those electrodes indicated in italicized bold lettering are those that simultaneously received local testosterone treatment.

TABLE 2. THRESHOLD VALUES

The threshold values in volts obtained for each of the three muscles stimulated during the experiment. Again, the large increase in threshold for MG becomes evident beginning at about day 22.

TABLE 3. TESTOSTERONE VALUES

The levels of serum testosterone from both cats in nmol/L. The sharp increase became evident on day 11 for both cats. Subsequent samples showed that lower levels of testosterone were circulating in the late stages of the experiment.

Figure 1.

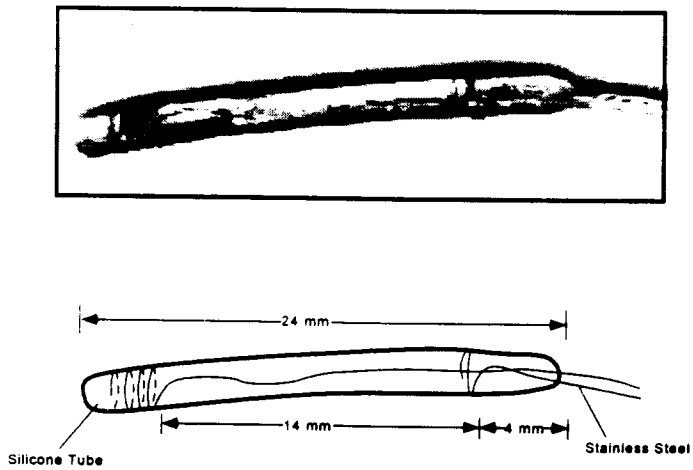
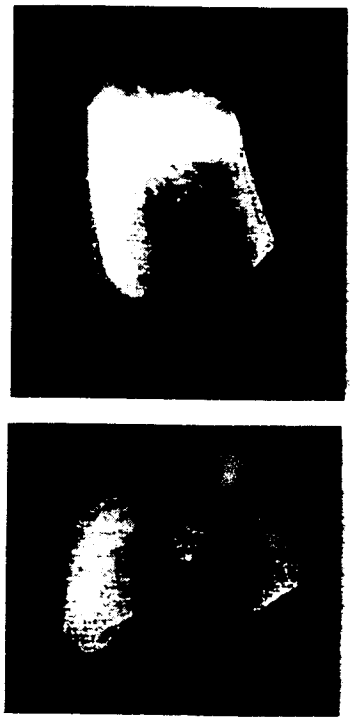


Figure 2.



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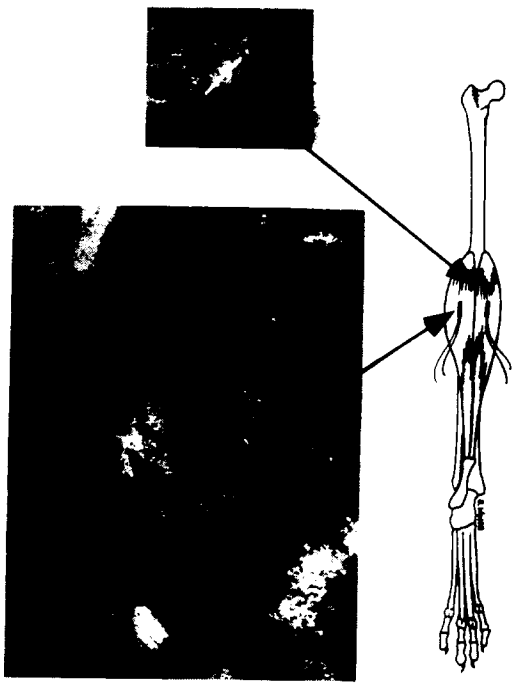


Figure 4.

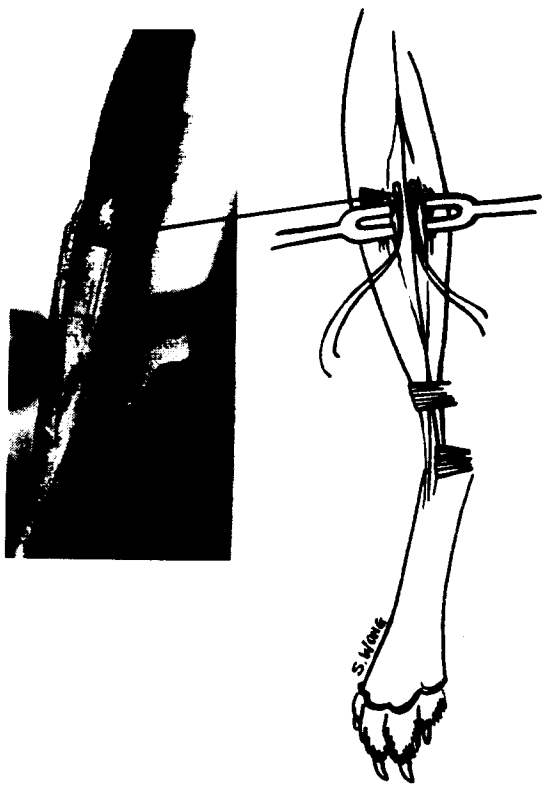
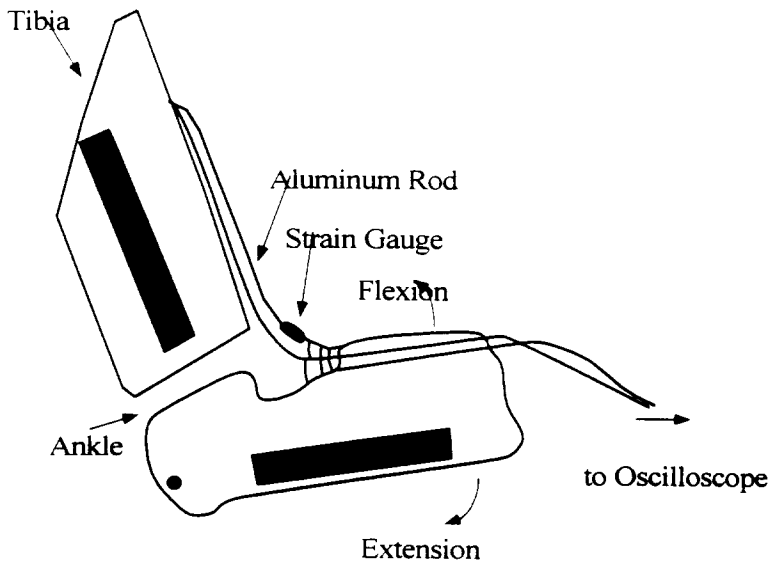


Table 1.

	CAT 1		CAT 2	
muscle	left	right	left	right
MG	***stim***	dummy	***dummy***	dummy
LG	***dummy***	dummy	***stim***	dummy
TA	***stim***	dummy	***dummy***	dummy
EDL	***dummy***	dummy	***stim***	dummy

Testosterone Treated Muscles

Figure 5.



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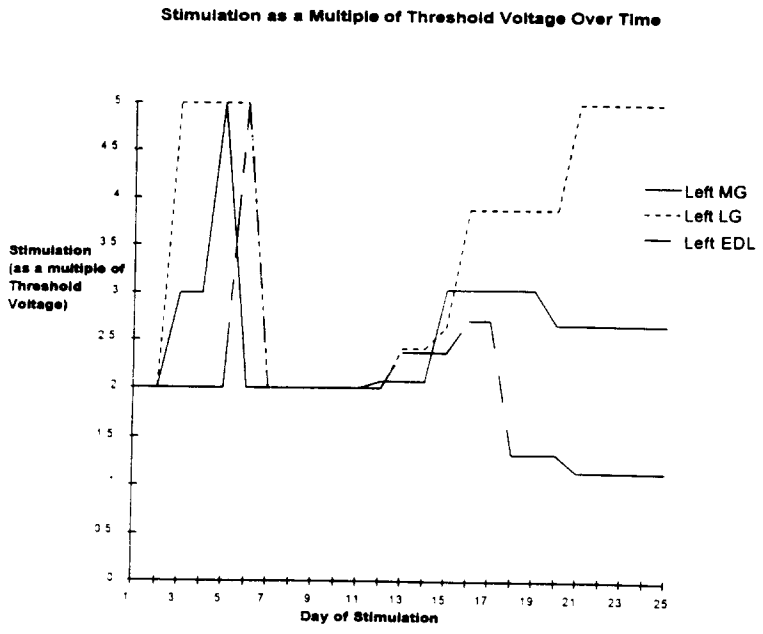

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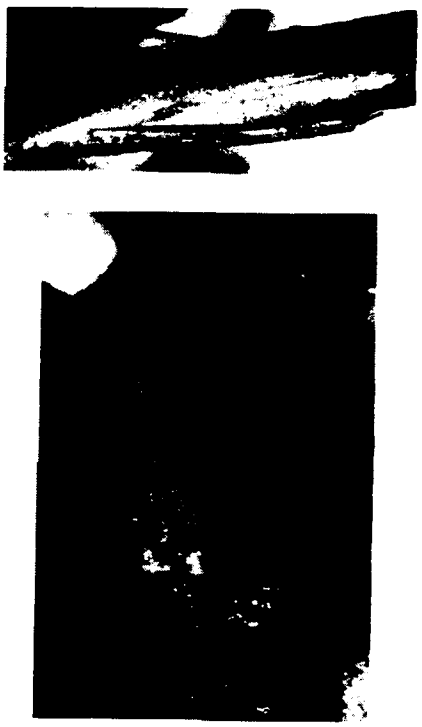

Figure 8.

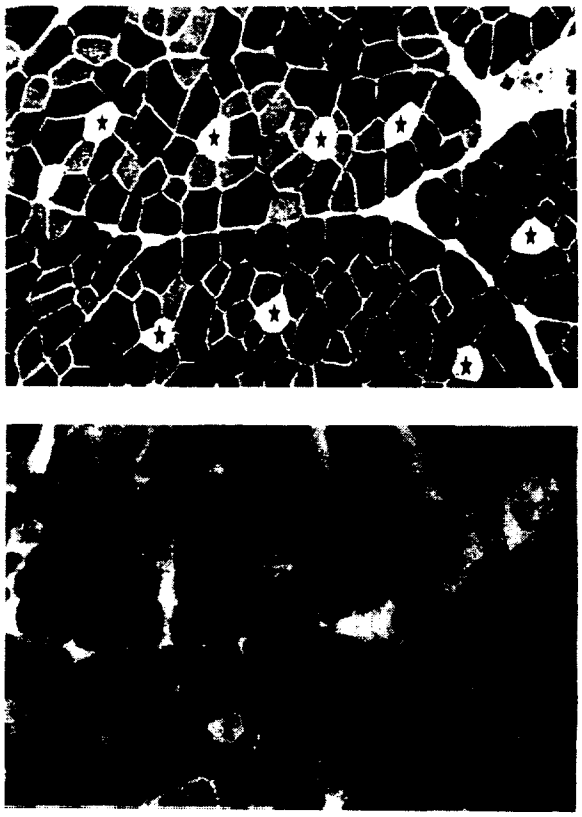

Figure 9.

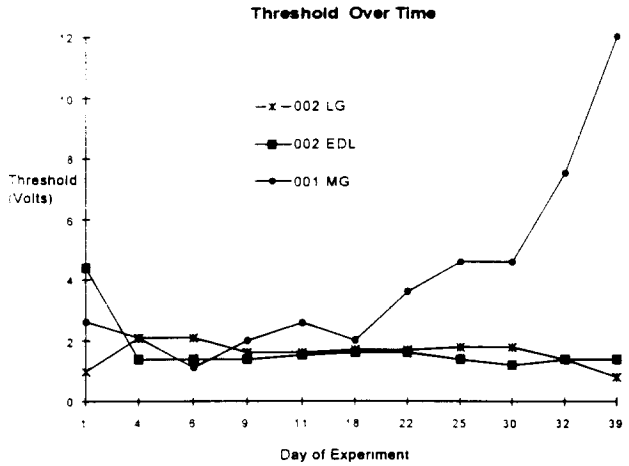

Table 2.

Threshold Measurements (Volts)

Day	Cat 2-LG	Cat 2-EDL	Cat 1-MG
1	1	4.4	2.64
4	2.1	1.4	2.1
6	2.1	1.4	1.1
9	1.6	1.4	2.0
11	1.6	1.5	2.6
18	1.7	1.6	2.0
22	1.7	1.6	3.6
26	1.8	1.4	4.6
30	1.8	1.2	4.6
32	1.4	1.4	7.5
39	0.8	1.4	12.0

Figure 10

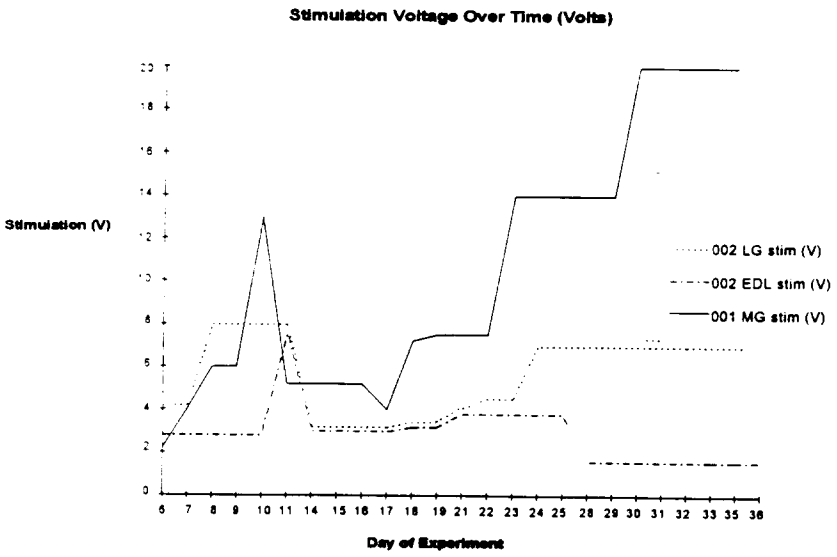

Figure 11.

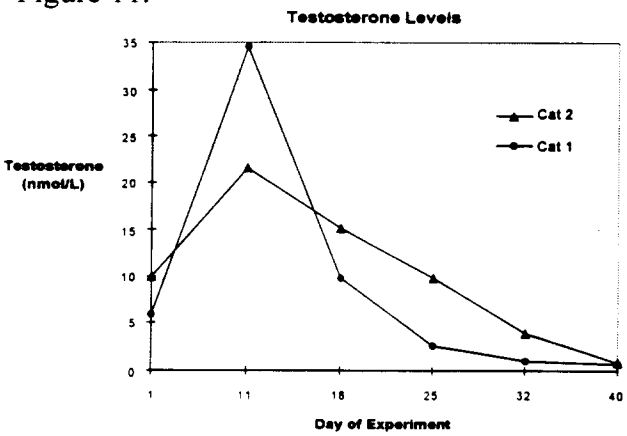

Table 3.

Serum Testosterone Levels(nmol/L)

Day	Cat 2	Cat 1
1	10.00	5.90
11	21.60	34.50
18	15.10	9.80
25	9.75	2.65
32	3.90	1.05
40	0.85	0.70

Figure 12.

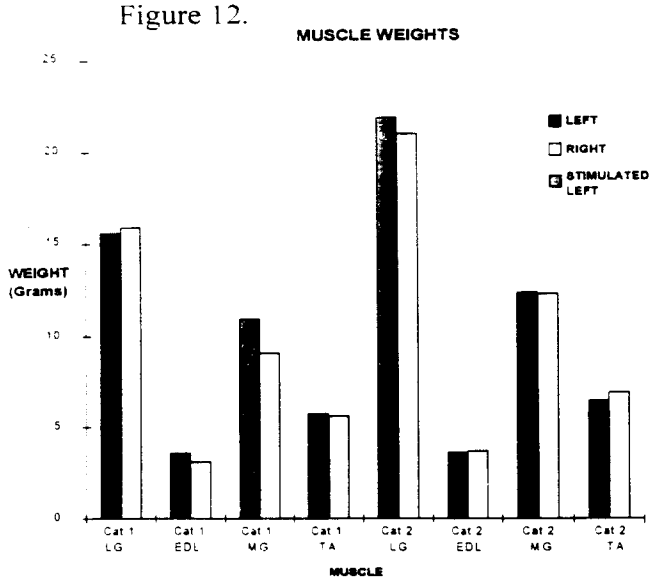

Figure 13.

Figure 14.

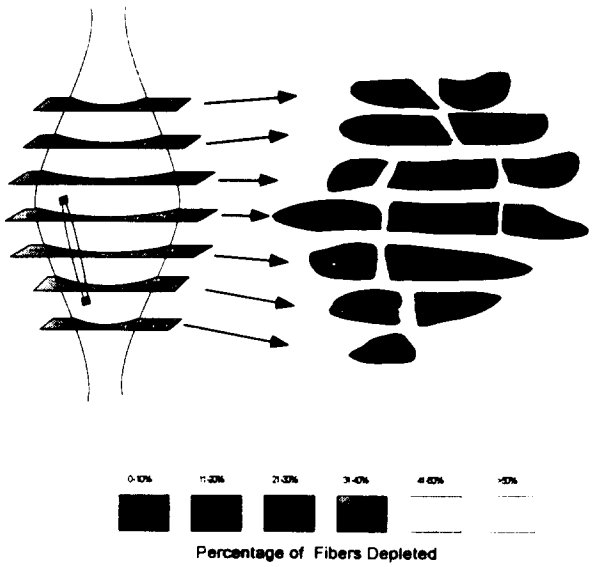

Figure 15.

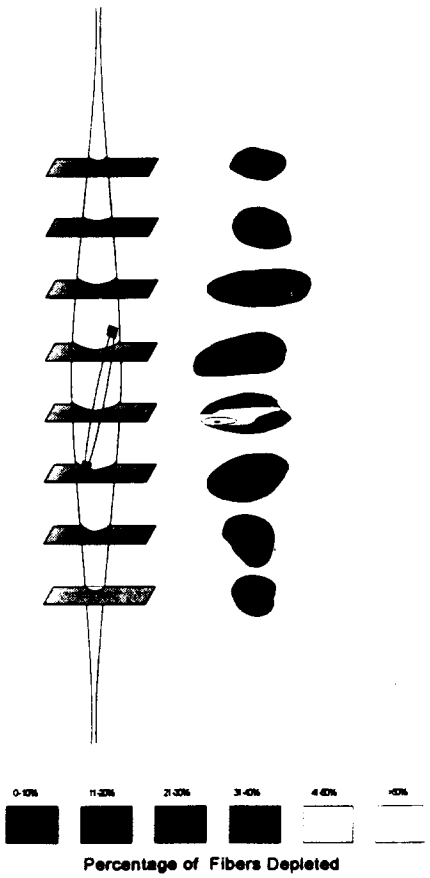

Figure 16.

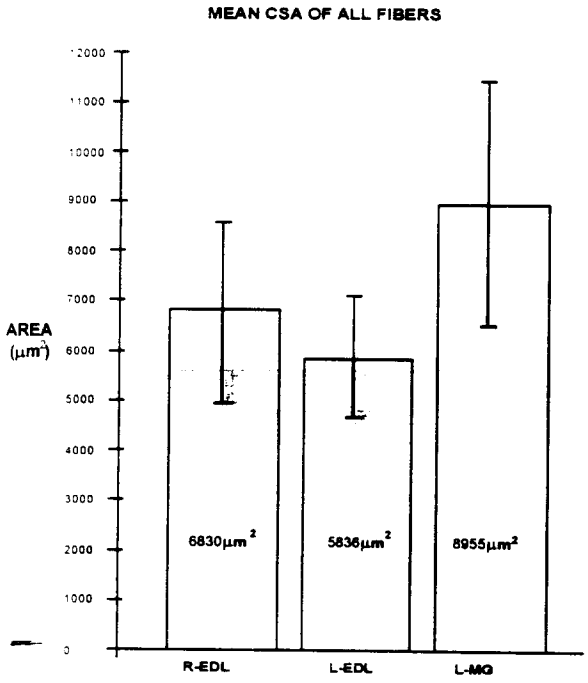

Figure 17.

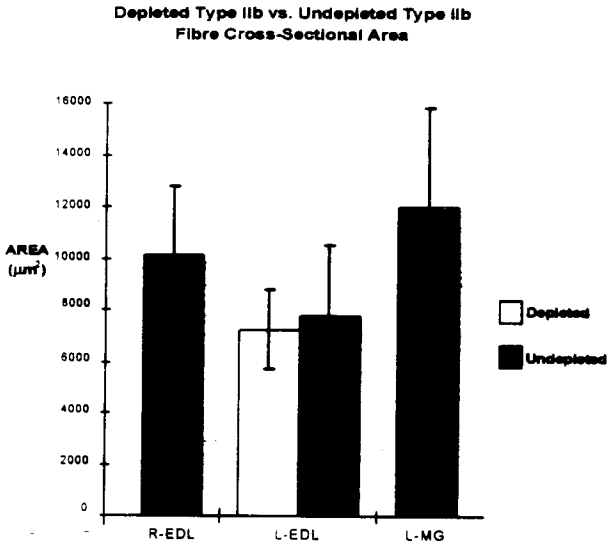

Figure 18.

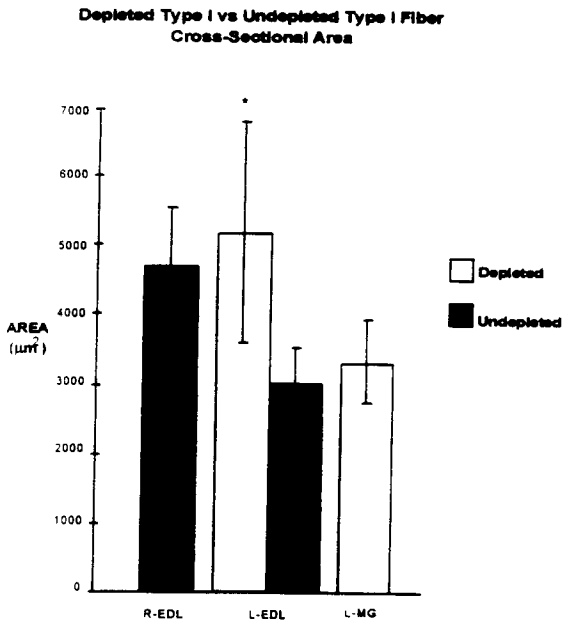

Figure 19.